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APPELLANTS:	Francisco Sanchez-Madrid, *et al.*		
SERIAL NUMBER:	10/770,639	EXAMINER:	Skelding, Zachary S.
FILING DATE:	February 2, 2004	ART UNIT:	1644
FOR:	IMMUNE REGULATION BASED ON THE TARGETING OF EARLY ACTIVATION MOLECULES		

MAIL STOP APPEAL BRIEF–PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPEAL BRIEF

Appellants file this Appeal Brief, in triplicate, pursuant to 37 C.F.R. § 41.37, in support of their Notice of Appeal, dated February 15, 2008. This Appeal Brief is timely filed with a petition for five-month extension and appropriate fee if submitted on or before on September 15, 2008. Applicant believes no additional fees are due. However, the Commissioner is hereby authorized to charge any fees that may be due, or credit any overpayment of same, to Deposit Account No. **50-0311**, Reference No. **27331-501 CIP2A**.

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I. Real Party In Interest

The real party in interest is Albor Biologics Inc, USA.

II. Related Appeals And Interferences

Appellants know of no other related appeals or interferences which will directly affect, be directly affected by, or have a bearing on the Board's decision in the pending appeal.

III. Status Of Claims

Pending claims 56, 59, 60, 67-69, and 105-108, as set forth in the Claims Appendix, are rejected and the subject of this appeal. Specifically, claims 56, 59, 60, 67-69 and 105-108 are rejected for allegedly being obvious over McInnes *et al.*, Immunol. Today 19(2):75-9 (Feb. 1998) ("McInnes 1998" or "McInnes #1") in light of Ledbetter *et al.* U.S. Publication No. 2003-0118592 ("Ledbetter") and McInnes *et al.*, Nat Med. 3(2):198-95 (Feb. 1997) ("McInnes 1997" or "McInnes #2").

Claims 70-77 have been withdrawn for allegedly being drawn to non-elected species pursuant to the Restriction Requirement of October 11, 2006. Claims 1-55, 57-58, 61-66, and 78-104 have been cancelled.

IV. Status Of Amendments

Appellants have filed this appeal from the examiner's August 15, 2007 Final Office Action ("the Final Office Action"). A Response and Amendment along with a Notice of Appeal was filed by Appellants on February 15, 2008. In the Advisory Action mailed on March 26, 2008 ("the Advisory Action"), the examiner indicated that this amendment would be entered.

V. Summary Of Claimed Subject Matter

Independent claim 56 recites a method of treating a subject having a disorder or condition characterized by an unwanted immune response comprising administering an effective amount of a depleting anti-CD69 antibody molecule, wherein the anti-CD69 antibody specifically binds SEQ ID NO:2 to the subject.

SEQ ID NO: 2 is an amino acid sequence of human CD69.

Depleting anti-CD69 antibody molecules are broadly referred to in the specification as "early activation molecule depletors" and are described in the specification as "antibody molecules which bind CD69 and deplete CD69 expressing cells."[1/] The specification also provides an example that distinguishes the *in vivo* effect of a depletor antibody from an antibody that merely antagonizes, neutralizes, or otherwise binds to CD69. Using a collagen-induced arthritis (CIA) model in DBA1 wild type mice, the specification shows that a depleting anti-CD69 antibody molecule ameliorates CIA *in vivo,* whereas a non-depleting anti-CD69 antibody molecule exacerbated CIA. Specially, Example 6 of the specification[2/] provides as follows (emphasis added):

> The effect of *in vivo* treatment with anti-CD69 antibody has been analyzed using two different anti-CD69 antibodies, mAb 2.2 and mAb 2.3, in the [collagen-induced arthritis (CIA)] model in DBA1 wild type mice.
>
> **MAb 2.2 behaves *in vitro* as a non-depletor antibody.** An IgG1, it is unable to bind complement and does not lyse CD69 expressing cells in an *in vitro* chromium assay (not shown). Furthermore, mAb 2.2 does not induce TGF-b synthesis *in vitro* in the absence of crosslinking (Esplugues et al. 2003. J. Exp. Med. 197:1093; Sancho 2003. J. Clin. Invest. 112:872).
>
> The effect of 2.2 anti-mouse CD69 antibody was analyzed *in vivo* in a model of CIA in DBA/1 mice. *In vivo* treatment with this antibody leads to the complete loss of expression of CD69 in populations that express the molecule, such as $CD3^{hi}$ thymocytes (FIG. 22). As shown in the upper right quadrant of the left panel, 14.2% of thymocytes express CD69. Following mAb 2.2 treatment, only 0.9% express CD69 (upper right quadrant, right panel). However, the total thymocyte pool remains constant, since the sums of the upper quadrants in each panel are the same, namely 20.7% (6.5+14.2 for control and 19.8+0.9 for treated). **This shows that mAb 2.2 does not mediate depletion of CD69+ cells *in vivo*. Further studies show that mAb 2.2 removes CD69 from the cell surface, i.e., antagonizes by down-modulation of CD69.**
>
> The treatment of CIA-induced DBA/1 mice with mAb 2.2 significantly exacerbated CIA when administered at days 20 and 28 during the initiation of the secondary response (FIG. 23), in agreement with our results in CD69-deficient mice (FIG. 1).
>
> * * *
>
> **MAb 2.3 behaves *in vitro* as a depletor antibody.** As an IgG2a, it binds complement and lyses CD69-expressing cells in an *in vitro* chromium assay (not shown).
>
> The effect of mAb 2.3 was also analyzed *in vivo* in a model of CIA in DBA/1 mice. *In vivo* treatment with this antibody leads to the depletion of CD69-expressing $CD3^{hi}$ thymocytes (FIG. 24). As shown in the upper right quadrant of the left panel, in this experiment 16.7% of thymocytes express CD69. Following mAb 2.3 treatment, only 0.1% express CD69 (upper right quadrant, right panel). However, the total thymocyte

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1/ *See e.g.*, Specification at page 7, beginning at line 22.

2/ Specification at page 104, line 14 to page 106, line 2.

> pool is strongly reduced, since the sums of the upper panels are now different, namely 24.8% in the control, but only 8.3% in the treated group. This shows that mAb 2.3 has depleted all CD69-expressing cells, rather than functionally `blocking` CD69.
>
> The treatment of CIA-induced DBA/1 mice with mAb 2.3 significantly reduced CIA when administered at days 20 and 28 during the initiation of the secondary response (FIG. 25).
>
> **These results show that the treatment with a down-modulating anti-CD69 could be useful to enhance certain immune responses. In contrast, the depletion of CD69 expressing cells may ameliorate diseases mediated by the activation of the immune system.**

The disorder or condition characterized by an unwanted immune response is described by the specification in the following: page 4, lines 18-23; page 6, lines 10-23; and dependent claims 67-69. In short, the unwanted immune response may be an acute or chronic inflammatory disorder or an immune disorder (*e.g.*, an autoimmune disorder). Specific diseases include rheumatoid arthritis, systemic lupus erythematosus, scleroderma, Sjögren syndrome, autoimmune diabetes, thyroiditis, or other organo-specific immune diseases, including psoriasis.

Dependent claim 60 (dependent from claim 59, which is dependent from claim 56) specifies a human anti-CD69 monoclonal antibody as the "depleting anti-CD69 antibody molecule" to be used in the method of claim 56. Human anti-CD69 monoclonal antibodies are described on pages 64 and 65 of the specification.

Independent claim 105 recites a method of treating a subject having a disorder or condition characterized by an unwanted immune response comprising administering to the subject an effective amount of a depleting anti-CD69 antibody molecule that specifically binds SEQ ID NO:2, wherein the depleting anti-CD69 antibody molecule may be conjugated to a second therapeutic agent. Thus, claim 105 differs from claim 56 in that it indicates that a second therapeutic agent may be conjugated to depleting anti-CD69 antibody molecule. Antibody molecule conjugates are described in the specification in the section beginning on page 68, line 11.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following issue is on appeal:

1. Whether the rejection of claims 56, 59, 60, 67-69 and 105-108 under 35 U.S.C. § 103(a) over McInnes 1998 in light of Ledbetter and McInnes 1997 was proper.

VII. ARGUMENT

A. The Teachings of McInnes 1998 in Light of Ledbetter And McInnes 1997 Do Not Render Obvious the Subject Matter of Claims 56-60, 67-69, and 105-108

The examiner has rejected claims 56-60, 67-69, and 105-108 over the teachings of McInnes 1998 in light Ledbetter and McInnes 1997. Appellants respectfully submit that this rejection is improper for the reasons set forth below.

1. *Summary of Cited References*

The primary reference, McInnes 1998, teaches that IL-15 can both recruit and expand CD45R0+ memory T-cells subsets in the synovial membrane, which, in the continued presence of IL-15 or via contact with macrophages, increases production of TNFα.[3/] To reduce TNFα production, McInnes 1998 suggests that IL-15 expression should be downgraded or IL-15 receptors should be targeted in order to decrease inflammation.[4/] McInnes 1998 does not suggest down regulation of CD69. McInnes 1998 does not suggest the use of a depleting anti-CD69 antibody molecule, and antibody that mediates depletion of CD69+ cells. No animal study data was shown in the teachings of McInnes 1998. Thus, McInnes 1998 does not disclose and does not enable a method of treating a subject with a depleting anti-CD69 antibody molecule of the claims.

Secondary reference of McInnes 1997 teaches that peripheral blood T-cells and U937 cells that are co-cultured in the presence of IL-15 *in vitro* have decreased TNFα production when treated with a neutralizing antibody to CD69.[5/] There are no teachings in McInnes 1997 that these antibodies deplete the cultures of $CD69^+$ cells. McInnes 1997 does not suggest down

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3/ *See* McInnes 1998 at page 77, column 1, first full paragraph.

4/ *See* McInnes 1998 at page 78, column 2, first full paragraph.

5/ *See* McInnes 1997 at page 193, Figure 7 legend.

regulation of CD69. McInnes 1997 does not suggest the use of a depleting anti-CD69 antibody molecule. No animal study data was shown in the teachings of McInnes 1997. Thus, McInnes 1997 does not disclose and does not enable a method of treating a subject with a depleting anti-CD69 antibody molecule of the claims.

The examiner acknowledges that neither McInnes 1998 nor McInnes 1997 teaches the use of a depleting anti-CD69 antibody to treat rheumatoid arthritis.[6/] McInnes 1997 discloses experiments performed using a "neutralizing antibodies to CD69".[7/] These are the same experiments relied upon in the primary reference of McInnes 1998. The McInnes references, therefore, never disclose depleting anti-CD69 antibodies. For this element, the examiner relies on the teachings of Ledbetter.

The secondary reference of Ledbetter teaches binding domain immunoglobulin fusion proteins.[8/] Specifically, Ledbetter teaches "binding domain-immunoglobulin fusion proteins that feature a binding domain for a cognate structure such as an antigen, a counterreceptor or the like, a wild-type IgG1, IgA or IgE hinge region polypeptide or a mutant IgG1 hinge region polypeptide having either zero, one or two cysteine residues, and immunoglobulin CH2 and CH3 domains."[9/] The antigen of the binding domain-immunoglobulin fusion protein of Ledbetter may be any one of CD19, CD20, CD22, CD37, CD40, L6, CD2, CD28, CD30, CD40, CD50 (ICAM3), CD54 (ICAM1), CD80, CD86, B7-H1, CD134 (OX40), CD137 (41BB), CD152 (CTLA-4), CD153 (CD30 ligand), CD154 (CD40 ligand), ICOS, CD19, CD3, CD4, CD25, CD8, CD11b, CD14, CD25, CD56, and CD69.[10/] Ledbetter teaches that any one of these molecules may be used in a method of treating a subject having or suspected of having a malignant condition or a B-cell disorder.[11/] Ledbetter provides no specific teaching of a depleting anti-CD69 antibody. Ledbetter provides no specific teaching of a depleting anti-CD69 monoclonal antibody. Ledbetter provides no specific teaching of a depleting anti-CD69 antibody for the treatment of rheumatoid arthritis.

6/ Final Office Action, page 5, fifth (5th) full paragraph.

7/ *See* McInnes 1997 at page 193, Figure 7 legend.

8/ *See* Ledbetter at the Abstract.

9/ *See* Ledbetter at the Abstract.

10/ *See e.g.*, Ledbetter at claim 17.

11/ *See* Ledbetter at paragraph [0038].

2. *The Examiner Has Failed to Establish a Prima Facie Case of Obviousness.*

Appellants submit that the examiner has failed to establish a *prima facie* case of obviousness. First, Appellants argue that the examiner is using an impermissible obvious to try standard. Second, Appellants show that there is no motivation to combine the teachings of McInnes 1998, Ledbetter, and McInnes 1997. Third, Appellants argue that one of ordinary skill in the art would not have a reasonable expectation of success in the methods of the instant claims in light of the teachings of McInnes 1998, Ledbetter, and McInnes 1997.

a. *The Examiner's Rejection is Based on an Impermissible Use of the Obvious to Try Standard*

The examiner is using an impermissible obvious to try standard in the rejection of claims 56, 59, 60, 67-69 and 105-108 over the teachings of McInnes 1998, Ledbetter, and McInnes 1997. An invention is not obvious if the inventor merely would have been motivated "to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical nor any direction as to which of many possible choices is likely to be successful."[12/] "Likewise, an invention would not be deemed obvious if all that was suggested 'was to explore a new technology or general approach that seemed to be a promising field of experimentation.'"[13/] In this regard, the teachings of McInnes 1998, Ledbetter and McInnes 1997, taken together, present numerous choices to be tried but do not lead one of ordinary skill in the art to a successful result.

The Federal Circuit has stressed that an invention is obvious when there is a lack of numerous parameters to vary and the prior art gives specific guidance as to how to reasonably achieve success.[14/] The Federal Circuit has consistently applied this principle in its decisions in In re O'Farrell, Pharmastem Therapeutics, Inc. v. Viacell, Inc., and Medichem, S.A. v. Rolabo, S.L. While the court found the inventions in those cases to be obvious, the facts of In Re O'Farrell, Pharmastem, and Medichem can be easily distinguished from the instant case.

12/ In re O'Farrell, 853 F.2d 894, 903 (Fed. Cir. 1988); *See also*, Pharmastem Therapeutics, Inc. v. Viacell, Inc., 491 F.3d 1342, 1364 (Fed. Cir. 2007) and Medichem, S.A. v. Rolabo, S.L., 437 F.3d 1157, 1166-67 (Fed. Cir. 2006).

13/ Pharmastem, 491 F.3d at 1364, quoting In re O'Farrell, 853 F.2d at 903; *See also* Medichem,. 437 F.3d at 1166-67.

14/ *See* In re O'Farrell, 853 F.2d at 903; Pharmastem, 491 F.3d at 1364; and Medichem, 437 F.3d at 1166-67.

In re O'Farrell involved an appeal from a decision of the U.S. Patent and Trademark Office Board of Patent Appeals and Interferences that rejected an application for obviousness.[15/] A year before they applied for a patent, two coinventors published an article that described the method for practicing their claimed invention and provided evidence suggesting the invention would be successful. In light of the article, the Federal Circuit held that their invention was obvious within the meaning of § 103.[16/]

The appellants in In re O'Farrell argued that at the time the article was published, there was enough unpredictability in the field of molecular biology to render their claimed method of synthesizing proteins nonobvious to one of ordinary skill in the art.[17/] In its discussion of the standard under § 103, the court noted that an invention could be "obvious to try" while ultimately remaining nonobvious.[18/] For example, an invention is nonobvious when it is discovered by "vary[ing] all parameters or try[ing] each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful."[19/] Similarly, an invention is nonobvious "where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it."[20/]

"For obviousness under § 103, all that is required is a reasonable explanation of success."[21/] In In re O'Farrell, the Federal Circuit found that the information in the prior art article provided such a reasonable expectation of success.[22/] As a result, the appellants' claimed invention was deemed obvious under § 103.

In Medichem, the Federal Circuit held that the addition of a tertiary amine to a patented reactive process was obvious in view of the prior art.[23/] Recognizing that the addition of the tertiary amine was the only significant difference between the appellee's and appellant's processes, the Federal Circuit noted that the appellee's invention was "a species within the genus

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15/ In re O'Farrell, 873 F.2d at 894.
16/ *Id.* at 904.
17/ *Id.* at 902.
18/ *Id.* at 903.
19/ *Id.*
20/ *Id.*
21/ *Id.*
22/ *Id.* at 904.
23/ Medichem, 437 F.3d at 1167.

of the [appellant's] invention."[24/] In Medichem, the court emphasized that "there [were] not numerous parameters to vary," nor was this a case "where the prior art [gave] merely general guidance."[25/] The guidance of the prior art in Medichem was "quite clear - namely, that . . . reactions of this kind can sometimes be optimized by adding low levels of a tertiary amine."[26/] As a result, the court concluded that the addition of a tertiary amine to the reactive process "would have been obvious in view of the [appellant's] patent and the prior art."[27/]

In Pharmastem, the Federal Circuit determined that patents for an umbilical cord banking process were obvious under the obvious to try standard.[28/] In that case, the two patents in question described "a process for collecting a newborn infant's umbilical cord blood at the time of birth, testing it for suitability for later use, preserving it through cryopreservation, and infusing it into an individual . . . whose hemotopoietic stem cells have been destroyed."[29/] Relying on the existence of prior art references (scientific articles that suggested using cord blood for this purpose and those that suggested cryopreservation and storage of the cord blood as needed), the court determined that "the idea of using cryopreserved cord blood to effect hematopoietic reconstitution was not new at the time the inventors filed" the patents in question.[30/]

While acknowledging that work of Pharmastem's inventors may have advanced the state of science in the field, the Federal Circuit concluded that the inventors "merely used routine research methods to prove what was already believed to be the case."[31/] In Pharmastem, there was "not an array of possible choices" as to how to achieve the scientific objective, nor were there "problems to be solved in implementing the prior art suggestion."[32/] To the contrary, the court found that "each step of the . . . [umbilical cord collection] procedure had been spelled out in the prior art."[33/] As a result, the Federal Circuit determined that it was unreasonable for a jury

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24/ Id. at 1161.
25/ Id. at 1167.
26/ Id.
27/ Id.
28/ Pharmastem Therapeutics, 491 F.3d at 1367.
29/ Id. at 1347.
30/ Id. at 1360.
31/ Id. at 1363.
32/ Id.
33/ Id.

to reach the conclusion that the patents were not obvious and reversed the district court's denial of JMOL on that issue.[34/]

Unlike the prior art references in Pharmastem, In re O'Farrell and Medichem, which pointed inventors to specific processes and limited the number of variables to try, the McInnes references do not limit an inventor to a list of potential therapeutic targets. Contrary to the examiner's assertion that the McInnes references "point one of ordinary skill in the art to three targets: the IL-15 ligand, the IL-15 receptor and CD69," nowhere do the references limit the potential therapeutic target to this list. The references do not even refer to CD69 as a therapeutic target; indeed, in the concluding section of McInnes 1998 under the heading *Therapeutic implications*, CD69 is not even mentioned. IL-15 is highlighted as the target. Specifically, McInnes 1998 states as follows:

> The identification of IL-15-mediated T-cell and monocyte activation in synovial membrane, apparently operating upstream from the effects of TNF-α, provides a novel target for such biological therapeutic approaches. This might be either through direct neutralization of IL-15 or by targeting IL-15 receptors, particularly IL-15Rα. Studies in animal models of arthritis are now required to address these exciting possibilities.

Thus, McInnes 1998 merely suggests IL-15 or one of the multitude of IL-15 receptors as potential therapeutic targets.

McInnes 1997 also does not identify CD69 as a therapeutic target. McInnes 1997 reports to the role of IL-15 in the induction of TNFα production in rheumatoid arthritis through activation of synovial T cells, which often express CD69, HLA-DR, and VLA1.[35/] The experimental result in McInnes 1997 shows that antibodies against CD69, LFA-1, and ICAM-1 significantly inhibited the ability of T cells to activate macrophages by cell contact, thereby implicating IL-15 as the source of the induction of TNFα production as opposed to other cytokines such as IL-2. Although CD69 is identified as a participant in the IL-15 molecular pathways, CD69 is not taught as a potential therapeutic target. Indeed, McInnes 1997 concludes with a list of implications of the results that in no way discloses CD69 as a potential target; all focus is placed on IL-15. This conclusion is evidenced by the fact that McInnes 1998, a review article published a year after McInnes 1997 (see excerpt above), fails to expressly identify CD69

34/ Id. at 1364.

35/ *See* McInnes 1997 at abstract and page 192, right column, last paragraph.

as a therapeutic target. Thus, even with the benefit of the data set forth in McInnes 1997, the main authors of McInnes 1997 failed to identify CD69 as a potential therapeutic target.

A suggestion that CD69 is part of the biological mechanism of a cytokine or cytokine receptor, such as IL-15 or IL-15 receptor, would not necessarily suggest to a person of ordinary skill in the art that CD69 is a target for therapy. Upregulation of CD69 has been suggested in response to other cytokines and in other cell types. For example, eosinophils, or white blood cells of the immune system, experience an upregulation in CD69 expression when incubated with IL-13, IL-4, or TNF-α.[36/] *In vivo* IL-12/GM-CSF treatment promotes a rapid up-regulation of CD69 on T cells.[37/] In short, CD69 and IL-15 are distinct molecules that play different and sometimes overlapping roles in the immune system. Suggesting IL-15 or IL-15 receptors as a possible target is not the same as suggesting the myriad of molecules implicated in the biological pathways of IL-15 or IL-15 receptors as possible targets.

Furthermore, even assuming, *arguendo*, that McInnes 1998 does suggest molecules other than IL-15 as possible therapeutic targets, the list of other possible targets would have to be expanded beyond CD69 to at least include:

1. IL-15;
2. the myriad of IL-15 receptors, particularly IL15Rα;
3. TNF-α;
4. leukocyte function-associated molecule 1 (LFA-1),
5. intercellular adhesion molecule (ICAM-1), and
6. CD69.

Each of these molecules are implicated by McInnes 1998 to be involved in the biological pathway that links IL15 expression to inflammation in rheumatoid arthritis.[38/] Thus, at best, the McInnes references only provide general guidance to a promising filed of experimentation and provide insight to a biological mechanism hypothesized to be involved in the inflammation seen in rheumatoid arthritis. Unlike the prior art reference in Pharmastem, which spelled out each

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36/ *See* Abstract of Luttmann *et al.*, "Synergistic Effects of Interleukin-4 or Interleukin-13 and Tumor Necrosis Factor-alpha on Eosinophil Activation *In Vitro*," AM. J. RESPIR. CELL MOL. BIOL., Volume 20, Number 3, March 1999 474-480.

37/ Kilinc *et al.*, "Reversing tumor immune suppression with intratumoral IL-12: activation of tumor-associated T effector/memory cells, induction of T suppressor apoptosis, and infiltration of CD8+ T effectors," J IMMUNOL. 2006 Nov 15;177(10):6962-73.

38/ *See e.g.*, McInnes 1998 at page 78, right column, first full paragraph and page 77, top of left column.

step of the procedure in question, the McInnes references point to IL-15 as the therapeutic target and implicate numerous other molecules as mediators of IL-15 activity.

With respect to Ledbetter, this reference discloses therapies for the depletion of B cells, and does not concern the T cell pathways shown in the McInnes references. Further, Appellants respectfully submit the Final Office Action mischaracterizes the teachings of Ledbetter. The examiner asserts in the Final Office Action at page 4 that "[a]s essentially stated in the prior Office Action of February 5, 2007, Ledbetter teaches human and humanized anti-CD69 antibodies with enhanced antibody dependent cell cytotoxicity and complement fixation activity, both of which lead to effective depletion of immune cells." The Office Action of February 5, 2007 at page 6 explains as follows:

> Ledbetter teaches human and humanized anti-CD69 antibodies with enhanced antibody dependent cell cytotoxicity and complement fixation activity, both of which lead to effective depletion of immune cells, such as B cells and T cells (see entire document, in particular pages 4-5, paragraphs [0021]-[0029], page 14, paragraph [0105] and claims 17 and 35). Ledbetter further teaches that radiolabeled antibodies and toxin conjugated antibodies are effective for treating tumors, such as B cell tumors (see in particular, pages 2-4, paragraphs [0011]-[0019]).

Contrary to the above characterization of the teachings of Ledbetter, Ledbetter does not explicitly disclose depleting anti-CD69 antibodies in these passages. Rather, Ledbetter at pages 4-5, paragraphs [0021]-[0029] provides a discussion related to CD20 antibodies and the depletion of B cells. Ledbetter at page 14, paragraph [0105] and claims 17 and 35 provides a long list of possible binding domain polypeptides and antigens, which include CD2, CD28, CD30, CD40, CD50 (ICAM3), CD54 (ICAM1), CD80, CD86, B7-H1, CD134 (OX40), CD137 (41BB), CD152 (CTLA-4), CD153 (CD30 ligand), CD154 (CD40 ligand), ICOS, CD19, CD20, CD22, CD37, L6, CD3, CD4, CD25, CD8, CD11b, CD14, CD25, CD56 and CD69. Ledbetter at pages 2-4, paragraphs [001]-[0019] merely provides a general discussion of immunoglobulin therapy. The examiner's reliance on these disclosures is not well understood by Appellants. Nowhere in these passages are depleting anti-CD69 antibodies expressly disclosed.

Rather, Ledbetter provides a general teaching concerning various cell surface antigens that may be targeted by the binding domain immunoglobulin fusion proteins.[39/] No specific data

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39/ Ledbetter at paragraph 105.

is provided regarding the production of antibodies or antibody-like molecules that specifically bind to CD69, or antibodies or antibody-like molecules that deplete CD69+ cells. Ledbetter mentions rheumatoid arthritis in a list of several pathologies that may be amenable to treatment using the myriad of antibodies or antibody-like molecules that specifically bind to one of many cell surface antigens. These antigens may be any one of CD19, CD20, CD22, CD37, CD40, L6, CD2, CD28, CD30, CD40, CD50 (ICAM3), CD54 (ICAM1), CD80, CD86, B7-H1, CD134 (OX40), CD137 (41BB), CD152 (CTLA-4), CD153 (CD30 ligand), CD154 (CD40 ligand), ICOS, CD19, CD3, CD4, CD25, CD8, CD11b, CD14, CD25, CD56, and CD69 .[40/]

Appellants assert that a person having ordinary skill in the art, reviewing the combination of McInnes 1998, Ledbetter, and McInnes 1997, would have to try each of numerous choices until he or she possibly arrived at a successful result. At most, McInnes 1998 and McInnes 1997 identify IL-15 and the several IL-15R as possible therapeutic targets; neither identifies CD69 as a therapeutic target. Ledbetter teaches antibody and antibody-derived molecules that bind to many cell surface antigens for the treatment of numerous pathologies. From these teachings, one of ordinary skill in the art would have to 1) choose between various molecules implicated in the IL-15 biological pathway (*e.g.*, IL-15, IL-15 receptors, TNF-α, LFA-1, ICAM-1, and CD69), 2) distinguish between various antibodies that bind specifically to CD69 and decrease expression of TNFα from depleting anti-CD69 antibodies, 3) identify and develop depleting antibodies, and 4) select depleting anti-CD69 antibodies over all of the other possible therapies for various immune conditions including rheumatoid arthritis. Therefore, these prior art teachings offer "merely general guidance," not specific guidance that would lead a person of ordinary skill in the art to the invention now claimed as an inventor would have to vary numerous parameters before achieving a successful result.[41/]

In light of the above, Appellants respectfully submit that the examiner is using an impermissible obvious to try standard in the rejection of claims 56, 59, 60, 67-69 and 105-108 over the teachings of McInnes 1998, Ledbetter, and McInnes 1997. Appellants respectfully request reversal of this rejection.

40/ Ledbetter at paragraphs 137-148.

41/ Medichem, 437 F.3d at 1167.

b. There is No Motivation to Combine the References

As stated by the Supreme Court, the framework for the objective analysis for determining obviousness under 35 U.S.C. 103 is stated in Graham v. John Deere Co.[42/] The factual inquiries enunciated by the Court are as follows:

(1) Determining the scope and content of the prior art;

(2) Ascertaining the differences between the claimed invention and the prior art; and

(3) Resolving the level of ordinary skill in the pertinent art.[43]

As is explained by the Federal Circuit, the motivation to combine is part of the discussion in determining the scope and content of the prior art,[44/] and where all claim limitations are found in a number of references, the factfinder must determine "[w]hat the prior art teaches... and whether it motivates a combination of teachings from different references."[45/] While the Court in KSR Int'l Co. v. Teleflex Inc. rejected a rigid application of the teaching, suggestion, or motivation ('TSM') test in an obviousness inquiry, the Court acknowledged the importance of identifying 'a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does' in an obviousness determination. Moreover, the [Supreme] Court indicated that there is "no necessary inconsistency between the idea underlying the TSM test and the Graham analysis."[46/]

Using the above as guidance, Appellants assert that one of ordinary skill in the art would not have had motivation to combine the teachings of McInnes 1998 with Ledbetter, nor would they have had motivation to combine the teachings of McInnes 1997 and Ledbetter. One of ordinary skill in the art could not find a motivation to combine McInnes 1998 with Ledbetter either in the references themselves or in knowledge generally available in the art. McInnes 1998

42/ 383 U.S. 1, 148 USPQ 459 (1966); reaffirmed and relied upon in KSR Int'l Co. v. Teleflex Inc., 127 S. Ct. 1727, 82 USPQ2d 1385 (2006).

43/ Graham v. John Deere Co., 383 U.S. 1, 148 USPQ 459 (1966); reaffirmed and relied upon in KSR Int'l Co. v. Teleflex Inc., 127 S. Ct. 1727, 82 USPQ2d 1385 (2006).

44/ DyStar Textilfarben GmbH & Co. Deutschland KG v. C.H. Patrick Co., 464 F.3d 1356, 1360, 80 USPQ2d 1641, 1645 (Fed. Cir. 2006); *citing* SIBIA Neurosciences, Inc. v. Cadus Pharma. Corp., 225 F.3d 1349, 1356 (Fed. Cir. 2000).

45/ Id. citing In re Fulton, 391 F.3d 1195, 1199-1200 (Fed. Cir. 2004).

46/ Takeda Chem. Indus., Ltd. v. Alphapharm Pty., Ltd., 492 F.3d 1350, 1356-1357 (Fed. Cir. 2007), quoting KSR, 127 S.Ct. at 1731.

teaches that CD69 is involved with IL-15 in the reduction of TNFα production by interacting with synovial T-cells. McInnes 1998 does not teach anything regarding the treatment of rheumatoid arthritis with depleting anti-CD69 antibodies. McInnes 1998 suggests that rheumatoid arthritis may be treated through the reduction of expression or activity of IL-15.[47/] McInnes 1998 does not teach or suggest that the depletion of CD69+ cells would be an effective treatment for rheumatoid arthritis. Thus, one of ordinary skill in the art would not be motivated to combine Ledbetter which teaches various depleting antibodies and related molecules for a large number of cell surface antigens, including CD69.

Likewise, one of ordinary skill in the art would not have had motivation to combine McInnes 1997 and Ledbetter. McInnes 1997 teaches that antibodies that bind to and neutralize CD69, but do not deplete CD69+ positive cells, reduce the amount of TNFα produced in *in vitro* co-cultures. There is no teaching that depleting CD69 antibodies are used to decrease TNFα production. McInnes 1997 does not teach that the depletion of CD69+ cells would be an effective treatment for rheumatoid arthritis. Thus, one of ordinary skill in the art would not have had motivation to combine McInnes 1997 with Ledbetter which teaches various depleting antibodies and related molecules for a large number of cell surface antigens.

Further, as indicated above, the immunoglobulin fusion proteins disclosed in Ledbetter are designed for uses involving B cell depletion such as B cell depletion therapies.[48/] Indeed, Ledbetter defines rheumatoid arthritis as a B-cell disorder.[49/] In this sense, Ledbetter actually teaches away from targeting antigens, such as CD69, that are present in both B cells and T cells as this may lead to a counterproductive immunosuppression.[50/] Specifically, Ledbetter provides as follows:[51/]

> Antigens that have been tested as targets for serotherapy of B and T cell malignancies include Ig idiotype, CD19, CD20, CD21, CD5, and CD52. Of these, the most success has been obtained using CD20 as a target for therapy of B cell lymphomas. Each of the other targets has been limited by the biological properties of the antigen. For example, surface idiotype can be altered through somatic mutation, allowing tumor cell escape. As other

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47/ *See e.g.*, McInnes 1998 at page 78, right column, first full paragraph.

48/ *Id.* at paragraph 137.

49/ See, *e.g.*, Ledbetter at paragraphs [0038] and [0043].

50/ *See* Ledbetter at paragraph [0017].

51/ *See* Ledbetter at paragraph [0017] (citations omitted)(emphasis added).

> examples, CD5, CD21, and CD19 are rapidly internalized after mAb binding, allowing tumor cells to escape destruction unless mAbs are conjugated with toxin molecules. CD22 is expressed on only a subset of B cell lymphomas, thereby limiting its usefulness, while *CD52 is expressed on both T cells and B cells and may therefore generate counterproductive immunosuppression by effecting selective T cell depletion.*

Thus, Ledbetter explains that targeting antigens that are expressed on both T cells and B cells (such as CD52, and likewise CD69[52/]) would actually be counterproductive in treating an unwanted immune response such as that seen in rheumatoid arthritis. Accordingly, a person of ordinary skill in the art would not have been motivated to combine the teachings of McInnes 1998 and McInnes 1997 with the teachings of Ledbetter to arrive at the present invention, because the use of the molecules of Ledbetter, which are designed to deplete B cells, in a method of depleting the CD69+ cells disclosed by McInnes 1998 and McInnes 1997 would lead to the undesirable and counterproductive immunosuppression.

In light of the above, Appellants respectfully submit that a person of ordinary skill in the art would not have been motivated to combine the teachings of McInnes 1998, Ledbetter, and McInnes 1997 in the manner proposed by the examiner. Thus, a person of ordinary skill in the art would not have combined these references to arrive at the subject matter of claims 56, 59, 60, 67-69 and 105-108. Appellants respectfully request reversal of this rejection.

c. The Examiner has Failed to Show a Reasonable Expectation of Success in Combining the Teachings of McInnes 1998 with Ledbetter and McInnes 1997

Appellants assert that a person having ordinary skill in the art reviewing the cited references would not have had a reasonable expectation of success at arriving at the methods of claims 56, 59, 60, 67-69 and 105-108, in part, because of the methods of the claims are based on unexpected results. Claims 56, 59, 60, 67-69 and 105-108 are presently being examined insofar as they encompass methods of treating rheumatoid arthritis.

One of ordinary skill in the art would not have a reasonable expectation of success regarding the methods of claims 56, 59, 60, 67-69 and 105-108 based on the teachings of McInnes 1998, Ledbetter, or McInnes 1997. None of McInnes 1998, Ledbetter, or McInnes 1997 shows any *in vivo* data from animal models for arthritis. The teachings of McInnes 1998,

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[52/] B-cells also express CD69. *See e.g.*, D'Arena *et al.*, "CD69 expression in B-cell chronic lymphocytic leukemia: a new prognostic marker?" HAEMATOLOGICA 2001 Sep;86(9):995-6.

Ledbetter, and McInnes 1997 have no specific evidence regarding the efficacy of depleting anti-CD69 antibodies. None of the cited references teaches a specific depleting anti-CD69 antibody, or shows any evidence of their efficacy in any context.

The Examiner cites additional references as further evidence that one of ordinary skill in the art would have had a reasonable expectation of success in combining the teachings of McInnes 1998, Ledbetter, and McInnes 1997. Appellants have reviewed the examiner's analysis of the cited references Sancho,[53/] Feng,[54/] Lauzurica,[55/] and Nakayama[56/] and cannot agree that any of these references support the examiner's conclusion that the above references render the present claims obvious. Rather, these references highlight the complexities of the field and merely identify promising areas for further experimentation.

As explained by the examiner, Sancho reports on the **"somewhat contradictory *in vitro* and *in vivo* results"** in the evidence suggesting a possible proinflammatory role for CD69. Specifically, Sancho at page 137, top left column, references Feng, Nakayama, and Lauzurica in the following passage:

> Despite the *in vitro* evidence suggesting a possible proinflammatory role for CD69, constitutive expression of CD69 by T cells in transgenic mice is not associated with inflammatory conditions. [Feng and Nakayama] Furthermore, analysis of antigen-specific response in mice has not revealed reduced T-Cell activation in the absence of CD69 [Lauzurica], suggesting that this receptor does not exert a net positive co-stimulatory effect in T cells *in vivo*, although a redundant role as a positive co-stimulus for T cells cannot be ruled out.

The examiner does not disagree with these conclusions of Sancho, but argues on pages 7 and 8 of the Office Action that these teachings **"do not particularly contradict"** the teachings of McInnes 1998 and McInnes 1997. While perhaps these teachings "do not particularly contradict" the teachings of McInnes 1998 and McInnes 1997, neither do they particularly support the examiner's conclusion that one of ordinary skill in art could have easily relied on the

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53/ Sancho *et al.*, "CD69 is an immunoregulatory molecule induced following activation," TRENDS IMMUNOL. 2005 Mar;26(3):136-40.

54/ Feng *et al.*, "A potential role for CD69 in thymocyte emigration," INT IMMUNOL. 2002 Jun;14(6):535-44.

55/ Lauzurica *et al.*, "Phenotypic and functional characteristics of hematopoietic cell lineages in CD69-deficient mice," BLOOD. 2000 Apr 1;95(7):2312-20.

56/ Nakayama *et al.*, "The generation of mature, single-positive thymocytes in vivo is dysregulated by CD69 blockade or overexpression," J IMMUNOL. 2002 Jan 1;168(1):87-94.

teachings these references to arrive at the present invention. Indeed, the examiner's analysis of Feng and Lauzurica highlights the complexities of the art and bolsters Appellants' position that a person of ordinary skill in the art would not have had a reasonable expectation of success in combining the teachings of McInnes 1998, Ledbetter, and McInnes 1997 to arrive at the present invention. Appellants respectfully maintain that one of ordinary skill in the art would not have a reasonable expectation of success in the methods of the instant claims in light of the teachings of McInnes 1998, Ledbetter, and McInnes 1997.

Further, Appellants respectfully submit that the examiner is carefully navigating the complexities of the art with the benefits of hindsight to arrive at the present invention and, moreover, understates the significance of the data presented in the specification. The instant specification shows for the first time the results of *in vivo* treatment of collagen induced arthritis (CIA) in mice with depleting anti-CD69 antibody.[57/] CIA is a widely accepted experimental model of inflammatory joint disease and specifically rheumatoid arthritis.[58/] McInnes 1998, Ledbetter, or McInnes 1997 show no experimental evidence of any sort that suggests that CD69 depleting antibodies work to alleviate the symptoms of rheumatoid arthritis. Thus, Appellants submit that without any data on point of any kind, and previously published contradictory *in vivo* data presented in the references cited by the examiner, the teachings of McInnes 1998, Ledbetter, and McInnes 1997 alone or in combination provide no reasonable expectation of success to one of ordinary skill in the art.

B. Any *Prima Facie* Case of Obviousness is Rebutted By Evidence of Unexpected Results Set Forth in the Specification

Appellants submit, as explained above, that the Examiner has not established a *prima facie* case of obviousness. However, even if the Examiner had presented such a case (Appellants submit that the Examiner did not present such a case), this case would be rebutted by the unexpected results presented in the instant application. As set forth in KSR, "combining

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57/ *See e.g.,* Specification from page 104, line 14 to page 106, line 2.

58/ *See e.g.*, Specification from page 30, line 30 to page 31, line 1 and Feldman *et al.* ANN REV. IMMUNOL. 14:397-440 (1996).

elements that work together 'in an unexpected and fruitful manner' would not have been obvious."[59/]

Appellants submit that the methods of claims 56, 59, 60, 67-69, and 105-108 are based upon unexpected results that shows that depleting anti-CD69 antibody molecules are effective in an *in vivo* model for unwanted immune response. The specification teaches, unexpectedly from the standpoint of one of ordinary skill in the art at the time the invention was made, that it is important that the CD69 specific antibody be a depletor of CD69+ cells, as opposed to specifically binding to CD69, while not depleting CD69+ cells in an *in vivo* model for unwanted immune response. Treatment of mice having an unwanted immune response (*i.e.*, collagen-induced arthritis (CIA)) with a CD69 specific antibody that does not deplete CD69+ cells *in vivo* (*i.e.*, mAb 2.2) actually exacerbated CIA in those mice.[60/] In contrast, treatment of CIA induced mice with a CD69 specific antibody that depletes CD69+ cells (*i.e.*, mAb 2.3) significantly reduced CIA.[61/] In this sense, the neutralizing antibodies of McInnes 1997 may actually exacerbate rheumatoid arthritis if they do not deplete CD69+ cells. This result was unexpected in light of McInnes 1998, Ledbetter, and McInnes 1997 and also the other previously published *in vivo* data present by the examiner. Thus, Appellants submit that the methods of claims 56, 59, 60, 67-69 and 105-108 are based on unexpected properties and thus are non-obvious over McInnes 1998, Ledbetter, and McInnes 1997.

The examiner rebuts the Appellants findings of unexpected results by citing McInnes 1998 on page 9 of the Office Action for its teaching that:

> T-cell-directed therapies that not only inhibit T-cell activation but also deplete T cells from the synovial compartment, or at least interfere with their membrane interactions, will probably be most efficacious.

McInnes 1998 suggests T-cell direct therapies that inhibit T-cell activation and also T-cell direct therapies that deplete T-cells or at least interfere with their membrane interactions. McInnes 1998, however, offers no specific or definite suggestion as to what therapy "will probably be most efficacious." As presented above, McInnes 1998 only suggests IL-15 or one of

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59/ KSR, 127 S. Ct. at 1740.

60/ *See e.g.*, Specification at page 105, lines 3-6.

61/ *See e.g.*, Specification at lines 27-29 and Figure 25.

the multitude of IL-15 receptors as potential therapeutic targets. Further, McInnes 1998 makes no distinction between therapies that inhibit T-cell activation from those therapies that would deplete T-cells. As pointed out by the present inventors, non-depletor CD69 antibodies that would merely neutralize T-cell activation may actually exacerbate an unwanted immune response. Thus, the present inventors were able to reach the conclusion that "treatment with a down-modulating anti-CD69 could be useful to enhance certain immune responses," while "the depletion of CD69 expressing cells may ameliorate diseases mediated by the activation of the immune system." [62/] These results are unexpected in view of this teaching in McInnes 1998 and would have been unexpected to one of ordinary skill in the art.

The examiner also rebuts the Appellants findings of unexpected results by citing Cheon[63/] on page 9 of the Final Office Action for its teaching that:

> TGF-β exerts diverse and even opposite effects depending on the cell types and conditions. In the present study, we provided evidence that TGF-β1 could contribute to the inflammation and progression of the disease in RA and OA.

This passage concerns TGF-β and does not contradict the applicant's unexpected findings that depleting anti-CD69 antibodies show unexpected properties. It appears from the above analysis that the examiner is conflating TGF-β and T-Cells with CD69. TGF-β, T-cells, and CD69 are all distinct compositions and play a distinct role in any normal or disease physiology. Under the examiner's rationale, Cheon suggests TGF-β as yet another therapeutic target for treating unwanted immune response. Appellants respectfully submit that Cheon, as well as all of the evidentiary references cited by examiner, provide only general teachings with respect to the subject matter of the instant invention. None of these evidentiary references set forth any specific teachings capable of refuting the evidence of unexpected results set forth in the specification.

As explained above, Appellants submit that the examiner's obviousness rejection is based on an improper obvious to try standard, that there is no motivation to combine McInnes 1998 and Ledbetter, or McInnes 1997, and that there is no reasonable expectation of success for combining

62/ Specification at page 104, line 14 to page 106, line 2 (Example 6).

63/ Cheon *et al.*, "Increased expression of pro-inflammatory cytokines and metalloproteinase-1 by TGF-beta1 in synovial fibroblasts from rheumatoid arthritis and normal individuals." CLIN EXP IMMUNOL. 2002 Mar; 127(3):547-52.

the teachings of McInnes 1998, Ledbetter, and McInnes 1997 to successfully arrive at the invention of claims 56, 59, 60, 67-69 and 105-108. Furthermore, even assuming, *arguendo*, that the examiner has set forth a *prima facie* case of obviousness, Appellants submit that the methods of claims 56, 59, 60, 67-69 and 105-108 are based on unexpected results. Thus, Appellants submit that claims 56, 59, 60, 67-69 and 105-108 are not obvious over McInnes 1998 in light of Ledbetter and McInnes 1997. Appellants respectfully request that this rejection be reversed.

C. The Combination of McInnes 1998, Ledbetter, and McInnes 1997 Fail to Suggest the Use of a Depleting Anti-CD69 Monoclonal Antibody as Set Forth in Claim 60

Appellants respectfully submit that the examiner fails to establish a *prima facie* case of obviousness with respect to the subject matter of claim 60. Claim 60 is dependent from claim 59, which is dependent from independent claim 56. Claim 60 states that the depleting anti-CD69 antibody molecule is a human anti-CD69 monoclonal antibody. None of McInnes 1998, Ledbetter, and McInnes 1997 provides for a human anti-CD69 monoclonal antibody.

The examiner acknowledges that neither McInnes 1998 nor McInnes 1997 teaches the use of a depleting anti-CD69 antibody to treat rheumatoid arthritis.[64/] McInnes 1997 discloses experiments performed using a "neutralizing antibodies to CD69".[65/] These are the same experiments relied upon in the primary reference of McInnes 1998. The McInnes references therefore never disclose depleting anti-CD69 antibodies, let alone depleting human anti-CD69 monoclonal antibody.

The secondary reference of Ledbetter teaches only binding domain-immunoglobulin fusion proteins, not depleting human anti-CD69 monoclonal antibodies.[66/] Specifically, Ledbetter teaches "binding domain-immunoglobulin fusion proteins that feature a binding domain for a cognate structure such as an antigen, a counterreceptor or the like, a wild-type IgG1, IGA or IgE hinge region polypeptide or a mutant IgG1 hinge region polypeptide having either zero, one or two cysteine residues, and immunoglobulin CH2 and CH3 domains."[67/] The

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64/ Final Office Action, page 5, fifth (5th) full paragraph.

65/ *See* McInnes 1997 at page 193, Figure 7 legend.

66/ *See* Ledbetter at the Abstract.

67/ *See* Ledbetter at the Abstract.

antigen of the binding domain-immunoglobulin fusion protein of Ledbetter may be any one of CD19, CD20, CD22, CD37, CD40, L6, CD2, CD28, CD30, CD40, CD50 (ICAM3), CD54 (ICAM1), CD80, CD86, B7-H1, CD134 (OX40), CD137 (41BB), CD152 (CTLA-4), CD153 (CD30 ligand), CD154 (CD40 ligand), ICOS, CD19, CD3, CD4, CD25, CD8, CD11b, CD14, CD25, CD56, and CD69.[68/] Fusion proteins are not the same as human monoclonal antibodies.

To establish a *prima facie* case of obviousness for a claimed invention, the cited references must teach or suggest all of the claim limitations.[69/] Here, the references, either alone or in combination, fail to teach depleting human anti-CD69 monoclonal antibodies.

The Federal Circuit has stated that '[n]ormally a *prima facie* case of obviousness is based upon structural similarity, *i.e.*, an established structural relationship between a prior art compound and the claimed compound.' That is so because close or established '[s]tructural relationships may provide the requisite motivation or suggestion to modify known compounds to obtain new compounds.'"[70/] Thus, "[a] known compound may suggest its homolog, analog, or isomer because such compounds 'often have similar properties and therefore chemists of ordinary skill would ordinarily contemplate making them to try to obtain compounds with improved properties.'"[71/] But, Deuel contains the caveat "that in order to find a *prima facie* case of unpatentability in such instances, a showing that the 'prior art would have suggested making the specific molecular modifications necessary to achieve the claimed invention' is also required.[72/]

The Federal Circuit confirmed that this test is consistent with KSR by noting that this "test for *prima facie* obviousness for chemical compounds is consistent with the legal principles enunciated in KSR. While the KSR Court rejected a rigid application of the teaching, suggestion, or motivation ('TSM') test in an obviousness inquiry, the Court acknowledged the

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68/ *See e.g.*, Id. at claim 17.

69/ *See* CFMT, Inc. v. YieldUp Int'l Corp., 349 F.3d 1333, 1342 (Fed. Cir. 2003); In re Royka, 490 F.2d 981, 985 (CCPA 1974) (obviousness requires a suggestion of all limitations in a claim).

70/ Takeda, 492 F.3d at 1355, quoting In re Deuel, 51 F.3d 1552, 1558 (Fed. Cir. 1995).

71/ Id.

72/ Takeda, 492 F.3d at 1356 (quoting Deuel, 51 F.3d at 1558, citing In re Jones, 958 F.2d 347 (Fed.Cir.1992); In re Dillon, 919 F.2d 688 (Fed. Cir. 1990); In re Grabiak, 769 F.2d 729, 731-32 (Fed. Cir. 1985); In re Lalu, 747 F.2d 703 (Fed.Cir.1984)).

importance of identifying 'a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does' in an obviousness determination. Moreover, the [Supreme] Court indicated that there is 'no necessary inconsistency between the idea underlying the TSM test and the Graham analysis.'"[73/] The court concluded that, "[t]hus, in cases involving new chemical compounds, it remains necessary to identify some reason that would have led a chemist to modify a known compound in a particular manner to establish *prima facie* obviousness of a new claimed compound."[74/]

Similarly, Appellants respectfully submit that the examiner has failed to set forth a reasonable rationale why a person of ordinary skill in the art would seek to obtain and use depleting human anti-CD69 monoclonal antibodies in the recited method. Monoclonal antibodies are structurally distinct from the fusion proteins of Ledbetter and the examiner provides no rationale for why a person of ordinary skill in the art would have modified the teachings of Ledbetter to arrive at the subject matter of claim 60. Appellants respectfully request that the rejection of claim 60 be reversed.

Respectfully submitted,

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73/ Takeda, 492 F.3d at 1356-1357, quoting KSR, 127 S.Ct. at 1731.

74/ Takeda, 492 F.3d at 1357.

VIII. CLAIMS APPENDIX

1-55. **(Canceled)**

56. **(Rejected; Previously Presented)** A method of treating a subject having a disorder or condition characterized by an unwanted immune response comprising administering an effective amount of a depleting anti-CD69 antibody molecule, wherein the anti-CD69 antibody specifically binds SEQ ID NO:2 to the subject.

57-58. **(Canceled)**

59. **(Rejected; Previously Presented)** The method of claim 56, wherein said depleting anti-CD69 antibody molecule selected from the group consisting of: a humanized anti-CD69 antibody molecule, a human anti-CD69 antibody molecule, a chimeric anti-CD69 antibody molecule and a deimmunized anti-CD69 antibody molecule.

60. **(Original)** The method of claim 59, wherein said human anti-CD69 antibody molecule is a monoclonal antibody.

61-66. **(Canceled)**

67. **(Rejected; Original)** The method of claim 56, wherein said disorder is an acute or chronic inflammatory disorder, or an immune disorder.

68. **(Rejected; Original)** The method of claim 67, wherein said disorder is an autoimmune disorder.

69. **(Rejected; Original)** The method of claim 56, wherein the disorder is selected from the group consisting of: rheumatoid arthritis, systemic lupus erythematosus, scleroderma, Sjögren syndrome, autoimmune diabetes, thyroiditis, and other organo-specific immune diseases, including psoriasis.

70. **(Withdrawn)** The method of claim 56, wherein the disorder is a neurological disorder, a gastrointestinal disorder, a cardiovascular disorder or a respiratory disorder.

71. **(Withdrawn)** The method of claim 70, wherein the disorder is a neurological disorder and the neurological disorder is selected from the group consisting of: multiple sclerosis, myasthenia gravis, and other neurological immune-mediated diseases.

72. **(Withdrawn)** The method of claim 70, wherein the disorder is a gastrointestinal disorder and the gastrointestinal disorder is selected from the group consisting of Crohn's disease, colitis, celiac disease, and hepatitis.

73. **(Withdrawn)** The method of claim 70, wherein the disorder is a respiratory disorder and the respiratory disorder is selected from the group consisting of: emphysema, and respiratory airways infections.

74. **(Withdrawn)** The method of claim 70, wherein the disorder is a cardiovascular disorder and the cardiovascular disorder is selected from the group consisting of: atherosclerosis, cardiomyopathy, rheumatic fever, endocarditis, vasculitis, and other immune-mediated diseases.

75. **(Withdrawn)** The method of claim 56, wherein the disorder is an allergic process or a hypersensitivity reaction (type I, II, III, and IV), including asthma, rhinitis, and other immune-mediated hypersensitivity reactions.

76. **(Withdrawn)** The method of claim 56, wherein the disorder is transplant or graft rejection.

77. **(Withdrawn)** The method of claim 56, wherein said disorder or condition is: acute lung injury, acute respiratory distress syndrome, asthma, bronchitis, cystic fibrosis, reperfusion injury, nephritis, pancreatitis, artery occlusion, stroke, transplantation, ultraviolet light induced injury, vasculitis, and sarcoidosis.

78-104. **(Canceled)**

105. **(Rejected; Previously Presented)** A method of treating a subject having a disorder or condition characterized by an unwanted immune response comprising administering an effective amount of a depleting anti-CD69 antibody molecule, wherein the anti-CD69 antibody specifically binds SEQ ID NO:2 to the subject, alone or conjugated to a second therapeutic agent.

106. **(Rejected; Original)** The method of claim 105, wherein said second therapeutic agent is selected from the group consisting of: chemotherapeutic agents; radioisotopes; and cytotoxins.

107. **(Rejected; Original)** The method of claim 105, wherein the antibody is a monoclonal antibody.

108. **(Rejected; Original)** The method of claim 107, wherein the monoclonal antibody is a human antibody.

IX. EVIDENCE APPENDIX

Evidence Entered by the Examiner

1. McInnes *et al.,* Immunol. Today 19(2):75-9 (Feb. 1998).
2. Ledbetter *et al.* U.S. Publication No. 2003-0118592.
3. McInnes *et al.,* Nat Med. 3(2):198-95 (Feb. 1997).
4. Feng *et al.*, "A potential role for CD69 in thymocyte emigration," INT IMMUNOL. 2002 Jun;14(6):535-44.
5. Lauzurica *et al.*, "Phenotypic and functional characteristics of hematopoietic cell lineages in CD69-deficient mice," BLOOD. 2000 Apr 1;95(7):2312-20.
6. Nakayama *et al.*, "The generation of mature, single-positive thymocytes in vivo is dysregulated by CD69 blockade or overexpression," J IMMUNOL. 2002 Jan 1;168(1):87-94.
7. Sancho *et al.*, "CD69 is an immunoregulatory molecule induced following activation," TRENDS IMMUNOL. 2005 Mar;26(3):136-40.
8. Cheon *et al.*, "Increased expression of pro-inflammatory cytokines and metalloproteinase-1 by TGF-beta1 in synovial fibroblasts from rheumatoid arthritis and normal individuals." CLIN EXP IMMUNOL. 2002 Mar; 127(3):547-52.

Evidence Relied Upon by Appellant

Cases

9. In re O'Farrell, 853 F.2d 894 (Fed. Cir. 1988).
10. Pharmastem Therapeutics, Inc. v. Viacell, Inc., 491 F.3d 1342 (Fed. Cir. 2007).
11. Medichem, S.A. v. Rolabo, S.L., 437 F.3d 1157 (Fed. Cir. 2006).
12. Graham v. John Deere Co., 383 U.S. 1, 148 USPQ 459 (1966).
13. KSR Int'l Co. v. Teleflex Inc., 127 S. Ct. 1727, 82 USPQ2d 1385 (2006).
14. DyStar Textilfarben GmbH & Co. Deutschland KG v. C.H. Patrick Co., 464 F.3d 1356, 80 USPQ2d 1641 (Fed. Cir. 2006).
15. SIBIA Neurosciences, Inc. v. Cadus Pharma. Corp., 225 F.3d 1349 (Fed. Cir. 2000).
16. In re Fulton, 391 F.3d 1195 (Fed. Cir. 2004).
17. Takeda Chem. Indus., Ltd. v. Alphapharm Pty., Ltd., 492 F.3d 1350 (Fed. Cir. 2007).
18. CFMT, Inc. v. YieldUp Int'l Corp., 349 F.3d 1333(Fed. Cir. 2003).

19. In re Royka, 490 F.2d 981 (CCPA 1974).
20. In re Deuel, 51 F.3d 1552 (Fed. Cir. 1995).
21. In re Jones, 958 F.2d 347 (Fed.Cir.1992).
22. In re Dillon, 919 F.2d 688 (Fed. Cir. 1990).
23. In re Grabiak, 769 F.2d 729 (Fed. Cir. 1985).
24. In re Lalu, 747 F.2d 703 (Fed.Cir.1984).

Articles

25. Abstract of Luttmann *et al.*, "Synergistic Effects of Interleukin-4 or Interleukin-13 and Tumor Necrosis Factor-alpha on Eosinophil Activation *In Vitro*," AM. J. RESPIR. CELL MOL. BIOL., Volume 20, Number 3, March 1999 474-480.
26. Kilinc *et al.*, "Reversing tumor immune suppression with intratumoral IL-12: activation of tumor-associated T effector/memory cells, induction of T suppressor apoptosis, and infiltration of CD8+ T effectors," J IMMUNOL. 2006 Nov 15;177(10):6962-73.
27. D'Arena *et al.*, "CD69 expression in B-cell chronic lymphocytic leukemia: a new prognostic marker?" HAEMATOLOGICA 2001 Sep;86(9):995-6.
28. Feldman *et al.* ANN REV. IMMUNOL. 14:397-440 (1996).

X. RELATED PROCEEDINGS APPENDIX

Appellants know of no other related appeals or interferences which will directly affect, be directly affected by, or have a bearing on the Board's decision in the pending appeal.

U.S. Appln No. 10/770,639
Reference No. 2

US 20030118592A1

(19) **United States**
(12) **Patent Application Publication** (10) **Pub. No.: US 2003/0118592 A1**
Ledbetter et al. (43) **Pub. Date: Jun. 26, 2003**

(54) **BINDING DOMAIN-IMMUNOGLOBULIN FUSION PROTEINS**

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(21) Appl. No.: **10/207,655**

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Related U.S. Application Data

(63) Continuation-in-part of application No. 10/053,530, filed on Jan. 17, 2002.

(60) Provisional application No. 60/367,358, filed on Jan. 17, 2001, now abandoned. Provisional application No. 60/385,691, filed on Jun. 3, 2002.

Publication Classification

(51) **Int. Cl.**[7] **A61K 39/395**; C07K 16/46
(52) **U.S. Cl.** **424/178.1**; 530/391.1

(57) **ABSTRACT**

The invention relates to novel binding domain-immunoglobulin fusion proteins that feature a binding domain for a cognate structure such as an antigen, a counterreceptor or the like, a wild-type IgG1, IGA or IgE hinge region polypeptide or a mutant IgG1 hinge region polypeptide having either zero, one or two cysteine residues, and immunoglobulin CH2 and CH3 domains, and that are capable of ADCC and/or CDC while occurring predominantly as polypeptides that are compromised in their ability to form disulfide-linked multimers. The fusion proteins can be recombinantly produced at high expression levels. Also provided are related compositions and methods, including cell surface forms of the fusion proteins and immunotherapeutic applications of the fusion proteins and of polynucleotides encoding such fusion proteins.

Figure 1A

2H7scFv-Ig cDNA and predicted amino acid sequence:

HindIII NcoI 2H7 V_L Leader Peptide→

M D F Q V Q I F S F L L I S A S

1 **AAGCTT**GCCG CC ATGGATTT TCAAGTGCAG ATTTTCAGCT TCCTGCTAAT CAGTGCTTCA

2H7 V_L→

V I I A R G Q I V L S Q S P A I L S A S

61 GTCATAATTG CCAGAGGACA AATTGTTCTC TCCCAGTCTC CAGCAATCCT GTCTGCATCT

P G E K V T M T C R A S S S V S Y M H W

121 CCAGGGGAGA AGGTCACAAT GACTTGCAGG GCCAGCTCAA GTGTAAGTTA CATGCACTGG

BamHI

Y Q Q K P G S S P K P W I Y A P S N L A

181 TACCAGCAGA AGCCAGGATC CTCCCCCAAA CCCTGGATTT ATGCCCCATC CAACCTGGCT

S G V P A R F S G S G S G T S Y S L T I

241 TCTGGAGTCC CTGCTCGCTT CAGTGGCAGT GGGTCTGGGA CCTCTTACTC TCTCACAATC

S R V E A E D A A T Y Y C Q Q W S F N P

301 AGCAGAGTGG AGGCTGAAGA TGCTGCCACT TATTACTGCC AGCAGTGGAG TTTTAACCCA

(Gly$_4$Ser)$_3$ Linker

P T F G A G T K L E L K G G G G S G G G

361 CCCACGTTCG GTGCTGGGAC CAAGCTGGAG CTGAAAGGTG GCGGTGGCTC GGGCGGTGGT

2H7 V_H→

G S G G G G S S Q A Y L Q Q S G A E L V

421 GGATCTGGAG GAGGTGGGAG CTCTCAGGCT TATCTACAGC AGTCTGGGGC TGAGCTGGTG

R P G A S V K M S C K A S G Y T F T S Y

481 AGGCCTGGGG CCTCAGTGAA GATGTCCTGC AAGGCTTCTG GCTACACATT TACCAGTTAC

N M H W V K Q T P R Q G L E W I G A I Y

541 AATATGCACT GGGTAAAGCA GACACCTAGA CAGGGCCTGG AATGGATTGG AGCTATTTAT

P G N G D T S Y N Q K F K G K A T L T V

601 CCAGGAAATG GTGATACTTC CTACAATCAG AAGTTCAAGG GCAAGGCCAC ACTGACTGTA

D K S S S T A Y M Q L S S L T S E D S A

661 GACAAATCCT CCAGCACAGC CTACATGCAG CTCAGCAGCC TGACATCTGA AGACTCTGCG

V Y F C A R V V Y Y S N S Y W Y F D V W

721 GTCTATTTCT GTGCAAGAGT GGTGTACTAT AGTAACTCTT ACTGGTACTT CGATGTCTGG

Figure 1B

```
                                          BclI
                                        ~~~~~~~human IgG1 Fc domain →
         G  T  G  T   T  V  T   V  S  D   Q  E  P  K   S  C  D   K  T  H
 781     GGCACAGGGA CCACGGTCAC CGTCTCTGAT CAGGAGCCCA AATCTTGTGA CAAAACTCAC

         T  C  P  P   C  P  A   P  E  L   L  G  G  P   S  V  F   L  F  P
 841     ACATGCCCAC CGTGCCCAGC ACCTGAACTC CTGGGGGGAC CGTCAGTCTT CCTCTTCCCC

         P  K  P  K   D  T  L   M  I  S   R  T  P  E   V  T  C   V  V  V
 901     CCAAAACCCA AGGACACCCT CATGATCTCC CGGACCCCTG AGGTCACATG CGTGGTGGTG

         D  V  S  H   E  D  P   E  V  K   F  N  W  Y   V  D  G   V  E  V
 961     GACGTGAGCC ACGAAGACCC TGAGGTCAAG TTCAACTGGT ACGTGGACGG CGTGGAGGTG

         H  N  A  K   T  K  P   R  E  E   Q  Y  N  S   T  Y  R   V  V  S
1021     CATAATGCCA AGACAAAGCC GCGGGAGGAG CAGTACAACA GCACGTACCG TGTGGTCAGC

         V  L  T  V   L  H  Q   D  W  L   N  G  K  E   Y  K  C   K  V  S
1081     GTCCTCACCG TCCTGCACCA GGACTGGCTG AATGGCAAGG AGTACAAGTG CAAGGTCTCC

         N  K  A  L   P  A  P   I  E  K   T  I  S  K   A  K  G   Q  P  R
1141     AACAAAGCCC TCCCAGCCCC CATCGAGAAA ACAATCTCCA AAGCCAAAGG GCAGCCCCGA

         E  P  Q  V   Y  T  L   P  P  S   R  D  E  L   T  K  N   Q  V  S
1201     GAACCACAGG TGTACACCCT GCCCCCATCC CGGGATGAGC TGACCAAGAA CCAGGTCAGC

         L  T  C  L   V  K  G   F  Y  P   S  D  I  A   V  E  W   E  S  N
1261     CTGACCTGCC TGGTCAAAGG CTTCTATCCC AGCGACATCG CCGTGGAGTG GGAGAGCAAT

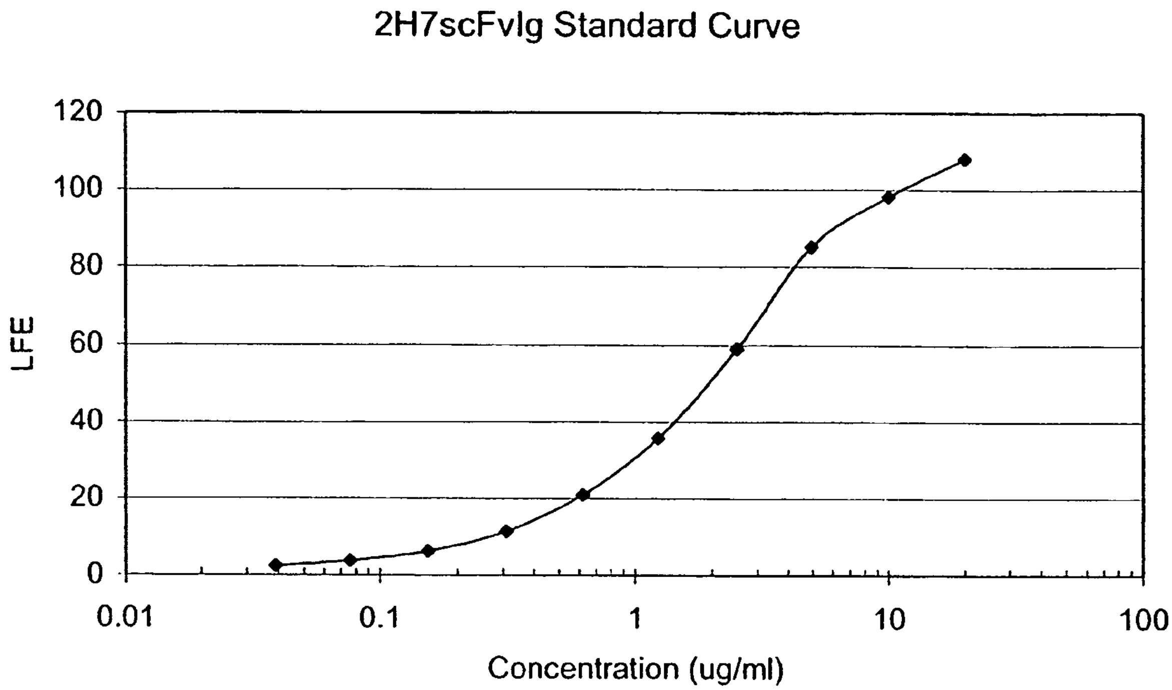
         G  Q  P  E   N  N  Y   K  T  T   P  P  V  L   D  S  D   G  S  F
1321     GGGCAGCCGG AGAACAACTA CAAGACCACG CCTCCCGTGC TGGACTCCGA CGGCTCCTTC

         F  L  Y  S   K  L  T   V  D  K   S  R  W  Q   Q  G  N   V  F  S
1381     TTCCTCTACA GCAAGCTCAC CGTGGACAAG AGCAGGTGGC AGCAGGGGAA CGTCTTCTCA

         C  S  V  M   H  E  A   L  H  N   H  Y  T  Q   K  S  L   S  L  S
1441     TGCTCCGTGA TGCATGAGGC TCTGCACAAC CACTACACGC AGAAGAGCCT CTCCCTGTCT

                        XbaI
                       ~~~~~
         P  G  K  *   S  R
1501     CCGGGTAAAT GATCTAGA
```

Figure 2



Clone	LFE @ 1:50	Estimated Concentration (μg/ml)
D2	26.1	56
IIIC6	25.7	55
IVA3	28.6	61
Spent bulk	29.6	64

Figure 3

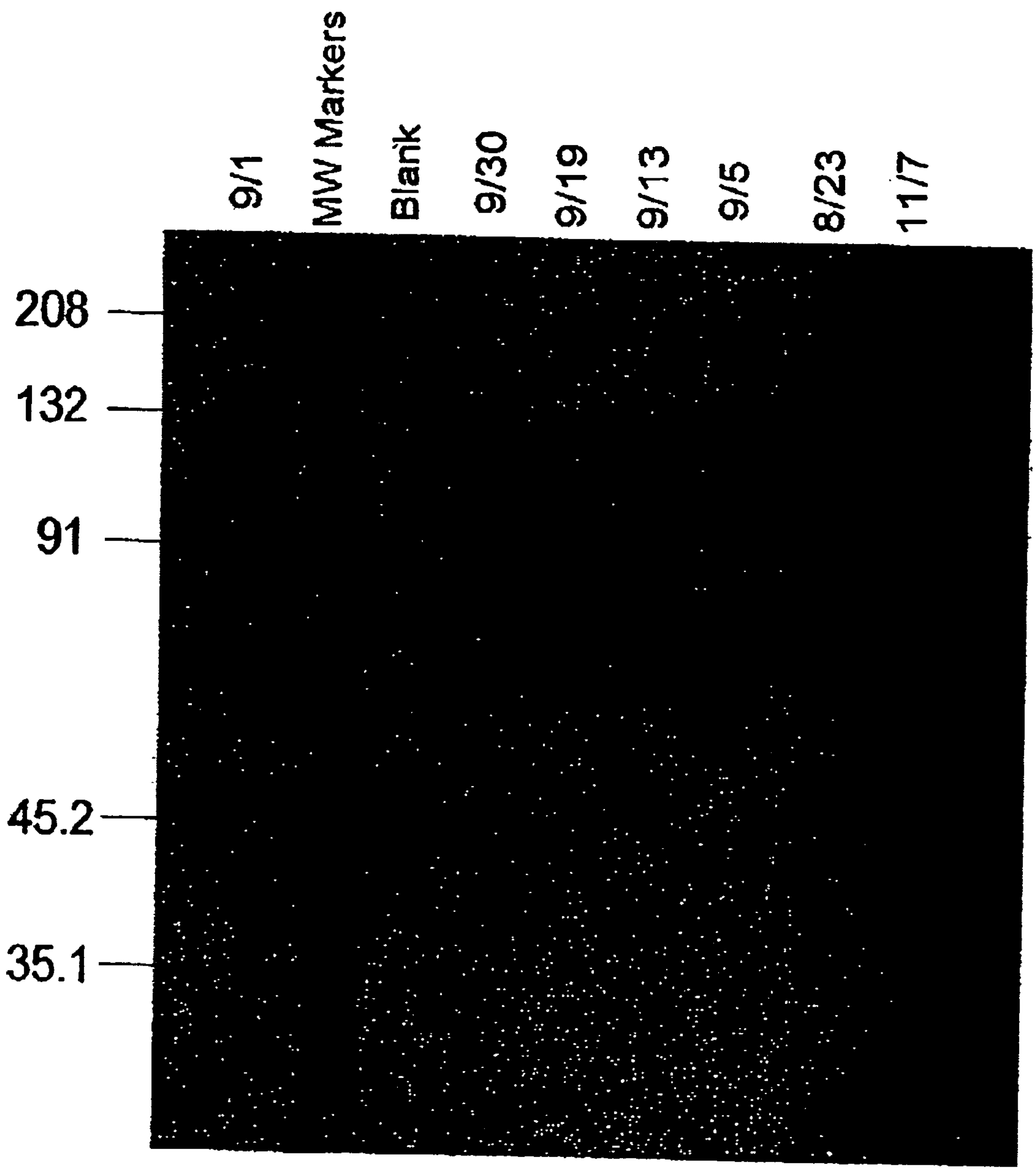


Figure 4A

Complement Mediated B Cell Killing After Binding of CD20-targeted 2H7 Derivatives:

2H7scFv-Ig Concentration	RAMOS	BJAB
20 μg/ml + complement	0.16	0.07
5 μg/ml + complement	0.2	N.D.
1.25 μg/ml + complement	0.32	0.1
Complement alone	0.98	0.94

*Viability was determined by trypan blue exclusion and is tabulated as the fraction of viable cells out of the total number of cells counted.

**N.D. (not determined).

Figure 4B

Antibody-dependent cellular cytotoxicity (ADCC) mediated by 2H7scFv-Ig:

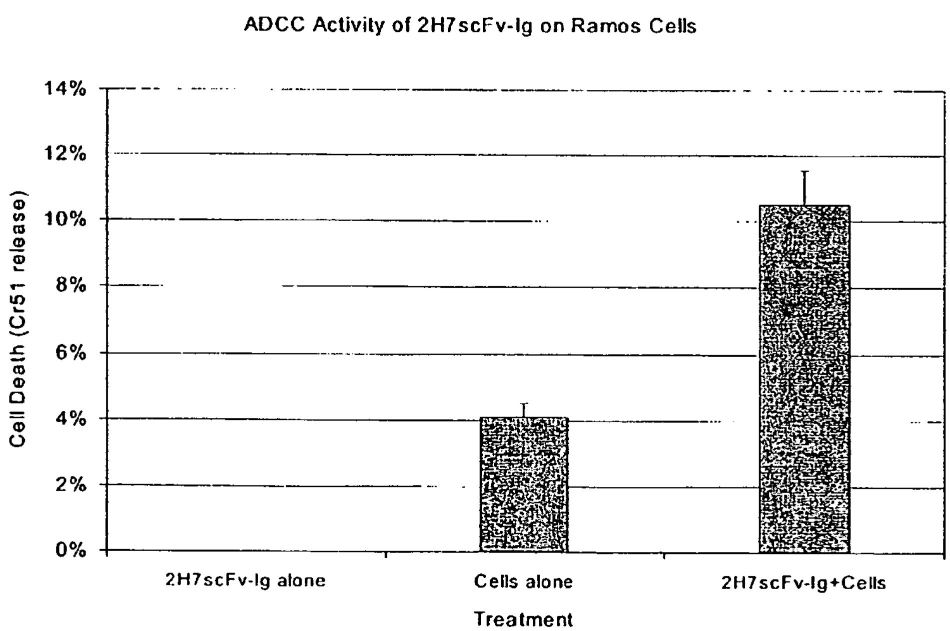


Figure 5

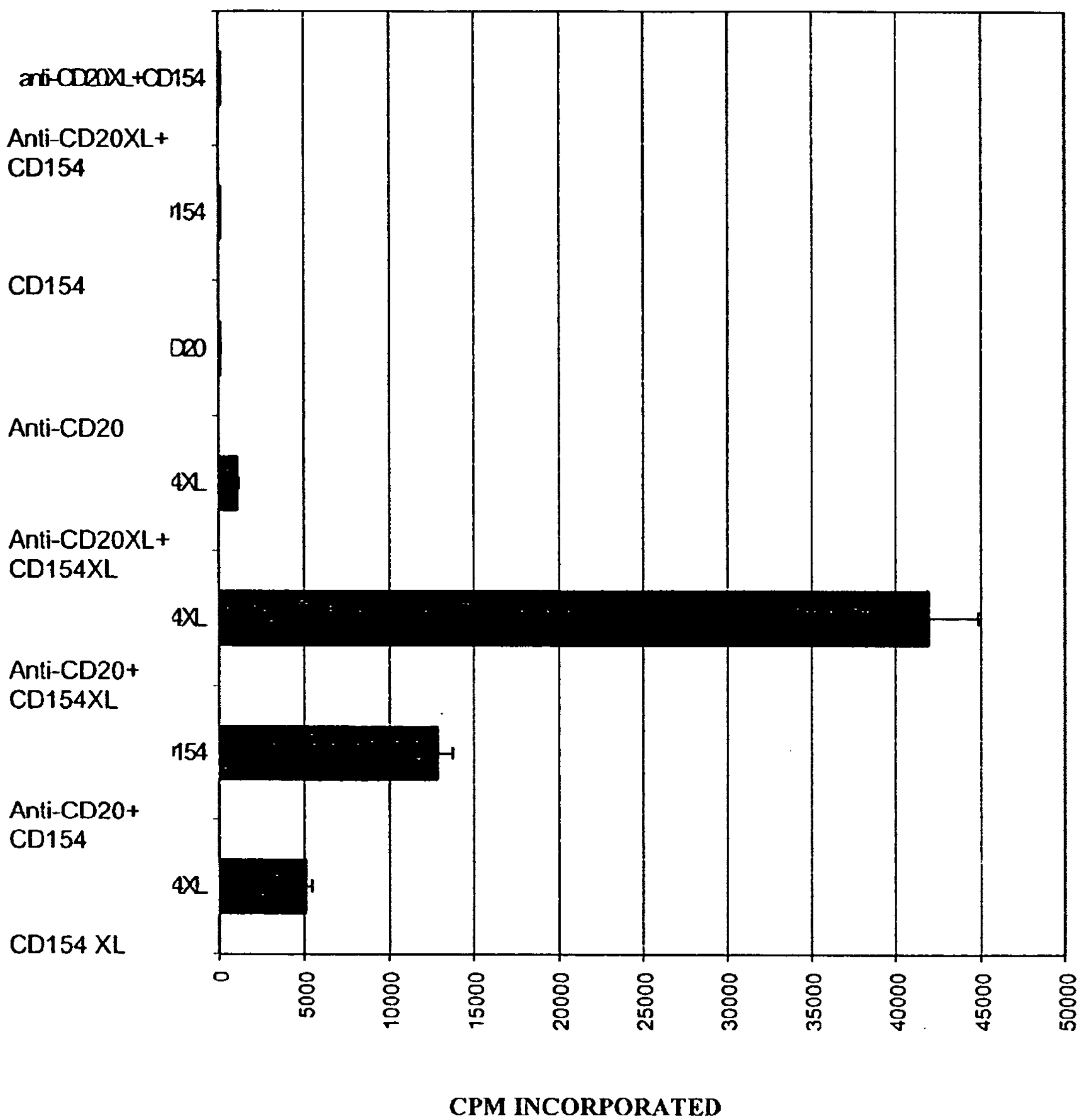

anti-CD20XL+CD154
Anti-CD20XL+
CD154
CD154
Anti-CD20
Anti-CD20XL+
CD154XL
Anti-CD20+
CD154XL
Anti-CD20+
CD154
CD154 XL
0
5000
10000
15000
20000
25000
30000
35000
40000
45000
50000
CPM INCORPORATED

Figure 6A and 6B

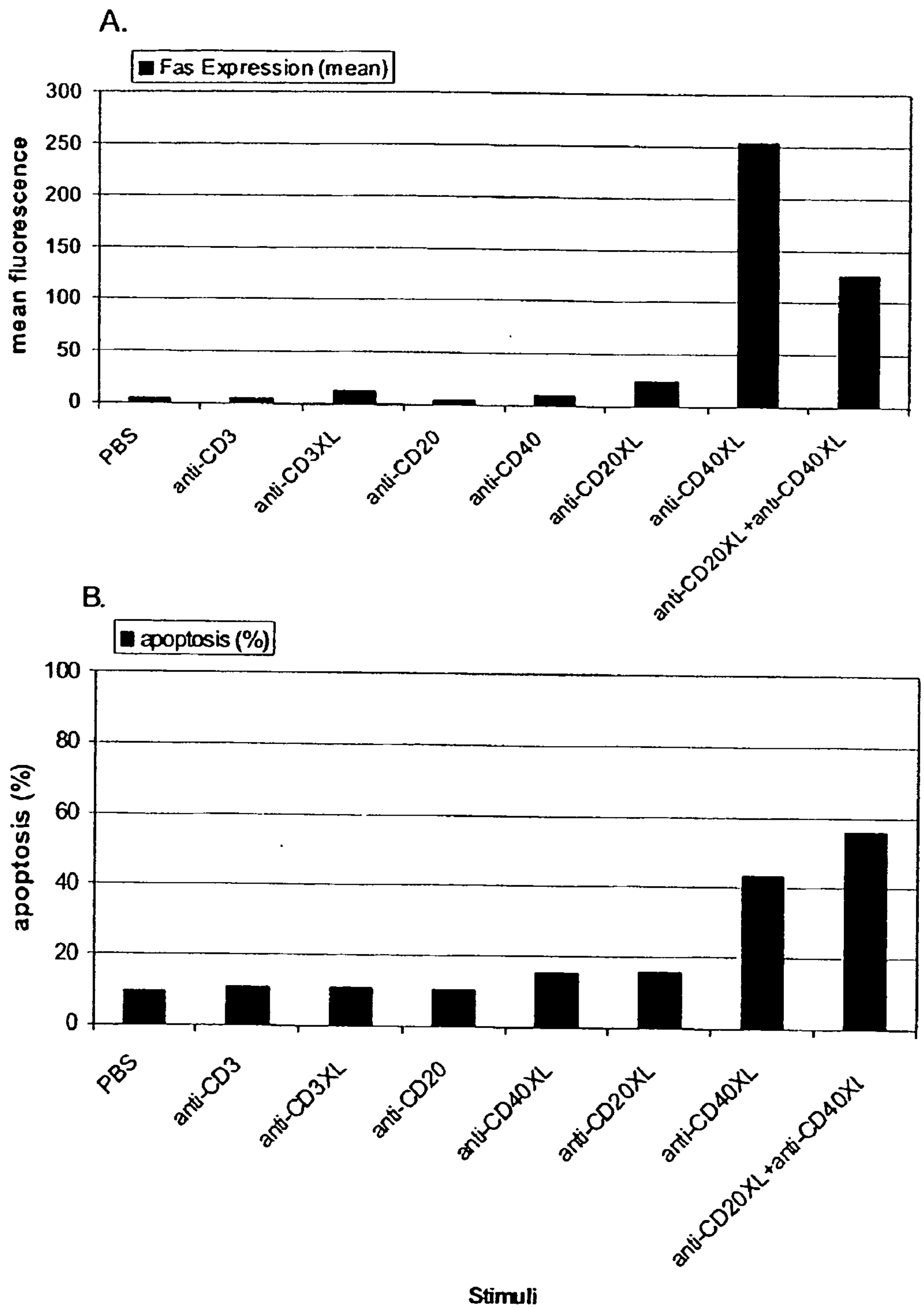


Figure 7A

2H7-CD154 L2 cDNA and predicted amino acid sequence:

```
      HindIII      NcoI  2H7 VL Leader Peptide ->
      ~~~~~~       ~~~~~~
                         M  D  F   Q  V  Q   I  F  S  F   L  L  I   S  A  S
  1   AAGCTTGCCG CC    ATGGATTT TCAAGTGCAG ATTTTCAGCT TCCTGCTAAT CAGTGCTTCA

                         2H7 VL ->
      V  I  I  A   R  G  Q   I  V  L   S  Q  S  P   A  I  L   S  A  S
 61   GTCATAATTG CCAGAGGACA AATTGTTCTC TCCCAGTCTC CAGCAATCCT GTCTGCATCT

      P  G  E  K   V  T  M   T  C  R   A  S  S  S   V  S  Y   M  H  W
121   CCAGGGGAGA AGGTCACAAT GACTTGCAGG GCCAGCTCAA GTGTAAGTTA CATGCACTGG

                       BamHI
                       ~~~~~~~
      Y  Q  Q  K   P  G  S   S  P  K   P  W  I  Y   A  P  S   N  L  A
181   TACCAGCAGA AGCCAGGATC CTCCCCCAAA CCCTGGATTT ATGCCCCATC CAACCTGGCT

      S  G  V  P   A  R  F   S  G  S   G  S  G  T   S  Y  S   L  T  I
241   TCTGGAGTCC CTGCTCGCTT CAGTGGCAGT GGGTCTGGGA CCTCTTACTC TCTCACAATC

      S  R  V  E   A  E  D   A  A  T   Y  Y  C  Q   Q  W  S   F  N  P
301   AGCAGAGTGG AGGCTGAAGA TGCTGCCACT TATTACTGCC AGCAGTGGAG TTTTAACCCA

                                          (Gly4Ser)3 Linker ->
      P  T  F  G   A  G  T   K  L  E   L  K  G  G   G  G  S   G  G  G
361   CCCACGTTCG GTGCTGGGAC CAAGCTGGAG CTGAAAGGTG GCGGTGGCTC GGGCGGTGGT

                                2H7 VH ->
      G  S  G  G   G  G  S   S  Q  A   Y  L  Q  Q   S  G  A   E  L  V
421   GGATCTGGAG GAGGTGGGAG CTCTCAGGCT TATCTACAGC AGTCTGGGGC TGAGCTGGTG

      R  P  G  A   S  V  K   M  S  C   K  A  S  G   Y  T  F   T  S  Y
481   AGGCCTGGGG CCTCAGTGAA GATGTCCTGC AAGGCTTCTG GCTACACATT TACCAGTTAC

      N  M  H  W   V  K  Q   T  P  R   Q  G  L  E   W  I  G   A  I  Y
541   AATATGCACT GGGTAAAGCA GACACCTAGA CAGGGCCTGG AATGGATTGG AGCTATTTAT

      P  G  N  G   D  T  S   Y  N  Q   K  F  K  G   K  A  T   L  T  V
601   CCAGGAAATG GTGATACTTC CTACAATCAG AAGTTCAAGG GCAAGGCCAC ACTGACTGTA

      D  K  S  S   S  T  A   Y  M  Q   L  S  S  L   T  S  E   D  S  A
661   GACAAATCCT CCAGCACAGC CTACATGCAG CTCAGCAGCC TGACATCTGA AGACTCTGCG

      V  Y  F  C   A  R  V   V  Y  Y   S  N  S  Y   W  Y  F   D  V  W
721   GTCTATTTCT GTGCAAGAGT GGTGTACTAT AGTAACTCTT ACTGGTACTT CGATGTCTGG
```

Figure 7B

human CD154/amino acid 48→

```
                                   Bcl/Bam hybrid site
      G  T  G  T   T  V  T   V  S  D   P  R  R  L   D  K  I   E  D  E
781   GGCACAGGGA CCACGGTCAC CGTCTCTGAT CCAAGAAGGT TGGACAAGAT AGAAGATGAA

      R  N  L  H   E  D  F   V  F  M   K  T  I  Q   R  C  N   T  G  E
841   AGGAATCTTC ATGAAGATTT TGTATTCATG AAAACGATAC AGAGATGCAA CACAGGAGAA

      R  S  L  S   L  L  N   C  E  E   I  K  S  Q   F  E  G   F  V  K
901   AGATCCTTAT CCTTACTGAA CTGTGAGGAG ATTAAAAGCC AGTTTGAAGG CTTTGTGAAG

                                                                  BclI
      D  I  M  L   N  K  E   E  T  K   K  E  N  S   F  E  M   Q  K  G
961   GATATAATGT TAAACAAAGA GGAGACGAAG AAAGAAAACA GCTTTGAAAT GCAAAAAGGT

      BclI
      ~~~~~
      D  Q  N  P   Q  I  A   A  H  V   I  S  E  A   S  S  K   T  T  S
1021  GATCAGAATC CTCAAATTGC GGCACATGTC ATAAGTGAGG CCAGCAGTAA AACAACATCT

      V  L  Q  W   A  E  K   G  Y  Y   T  M  S  N   N  L  V   T  L  E
1081  GTGTTACAGT GGGCTGAAAA AGGATACTAC ACCATGAGCA ACAACTTGGT AACCCTGGAA

      N  G  K  Q   L  T  V   K  R  Q   G  L  Y  Y   I  Y  A   Q  V  T
1141  AATGGGAAAC AGCTGACCGT TAAAAGACAA GGACTCTATT ATATCTATGC CCAAGTCACC

                            HindIII
                            ~~~~~~~
      F  C  S  N   R  E  A   S  S  Q   A  P  F  I   A  S  L   C  L  K
1201  TTCTGTTCCA ATCGGGAAGC TTCGAGTCAA GCTCCATTTA TAGCCAGCCT CTGCCTAAAG

      S  P  G  R   F  E  R   I  L  L   R  A  A  N   T  H  S   S  A  K
1261  TCCCCCGGTA GATTCGAGAG AATCTTACTC AGAGCTGCAA ATACCCACAG TTCCGCCAAA

      P  C  G  Q   Q  S  I   H  L  G   G  V  F  E   L  Q  P   G  A  S
1321  CCTTGCGGGC AACAATCCAT TCACTTGGGA GGAGTATTTG AATTGCAACC AGGTGCTTCG

                                                NcoI
                                               ~~~~~~~
      V  F  V  N   V  T  D   P  S  Q   V  S  H  G   T  G  F   T  S  F
1381  GTGTTTGTCA ATGTGACTGA TCCAAGCCAA GTGAGCCATG GCACTGGCTT CACGTCCTTT

                        XhoI       XbaI
                       ~~~~~~     ~~~~
      G  L  L  K   L  E  *   *  S  R
1441  GGCTTACTCA AACTCGAGTG ATAATCTAGA
```

Figure 7C

2H7scFv-CD154 S4 cDNA and predicted amino acid sequence:

```
      HindIII     NcoI
      ~~~~~~      ~~~~~~2H7 VL Leader Peptide→
                        M  D  F  Q  V  Q  I  F  S  F  L  L  I  S  A  S
  1   AAGCTTGCCG CC  ATGGATTT TCAAGTGCAG ATTTTCAGCT TCCTGCTAAT CAGTGCTTCA

                       2H7 VL →
      V  I  I  A  R  G  Q  I  V  L  S  Q  S  P  A  I  L  S  A  S
 61   GTCATAATTG CCAGAGGACA AATTGTTCTC TCCCAGTCTC CAGCAATCCT GTCTGCATCT

      P  G  E  K  V  T  M  T  C  R  A  S  S  S  V  S  Y  M  H  W
121   CCAGGGGAGA AGGTCACAAT GACTTGCAGG GCCAGCTCAA GTGTAAGTTA CATGCACTGG

                     BamHI
                     ~~~~~~~
      Y  Q  Q  K  P  G  S  S  P  K  P  W  I  Y  A  P  S  N  L  A
181   TACCAGCAGA AGCCAGGATC CTCCCCCAAA CCCTGGATTT ATGCCCCATC CAACCTGGCT

      S  G  V  P  A  R  F  S  G  S  G  S  G  T  S  Y  S  L  T  I
241   TCTGGAGTCC CTGCTCGCTT CAGTGGCAGT GGGTCTGGGA CCTCTTACTC TCTCACAATC

      S  R  V  E  A  E  D  A  A  T  Y  Y  C  Q  Q  W  S  F  N  P
301   AGCAGAGTGG AGGCTGAAGA TGCTGCCACT TATTACTGCC AGCAGTGGAG TTTTAACCCA

                                          (Gly4Ser)3 Linker →
      P  T  F  G  A  G  T  K  L  E  L  K  G  G  G  G  S  G  G  G
361   CCCACGTTCG GTGCTGGGAC CAAGCTGGAG CTGAAAGGTG GCGGTGGCTC GGGCGGTGGT

                             2H7 VH →
      G  S  G  G  G  G  S  S  Q  A  Y  L  Q  Q  S  G  A  E  L  V
421   GGATCTGGAG GAGGTGGGAG CTCTCAGGCT TATCTACAGC AGTCTGGGGC TGAGCTGGTG

      R  P  G  A  S  V  K  M  S  C  K  A  S  G  Y  T  F  T  S  Y
481   AGGCCTGGGG CCTCAGTGAA GATGTCCTGC AAGGCTTCTG GCTACACATT TACCAGTTAC

      N  M  H  W  V  K  Q  T  P  R  Q  G  L  E  W  I  G  A  I  Y
541   AATATGCACT GGGTAAAGCA GACACCTAGA CAGGGCCTGG AATGGATTGG AGCTATTTAT

      P  G  N  G  D  T  S  Y  N  Q  K  F  K  G  K  A  T  L  T  V
601   CCAGGAAATG GTGATACTTC CTACAATCAG AAGTTCAAGG GCAAGGCCAC ACTGACTGTA

      D  K  S  S  S  T  A  Y  M  Q  L  S  S  L  T  S  E  D  S  A
661   GACAAATCCT CCAGCACAGC CTACATGCAG CTCAGCAGCC TGACATCTGA AGACTCTGCG

      V  Y  F  C  A  R  V  V  Y  Y  S  N  S  Y  W  Y  F  D  V  W
721   GTCTATTTCT GTGCAAGAGT GGTGTACTAT AGTAACTCTT ACTGGTACTT CGATGTCTGG
```

Figure 7D

human CD154/amino acid 108 →

```
                              Bcl/Bam hybrid site                  BclI
       G  T  G  T   T  V  T   V  S  D   P  E  N  S   F  E  M   Q  K  G
781    GGCACAGGGA CCACGGTCAC CGTCTCTGAT CCAGAAAACA GCTTTGAAAT GCAAAAAGGT

       BclI
       ~~~~~
       D  Q  N  P   Q  I  A   A  H  V   I  S  E  A   S  S  K   T  T  S
841    GATCAGAATC CTCAAATTGC GGCACATGTC ATAAGTGAGG CCAGCAGTAA AACAACATCT

       V  L  Q  W   A  E  K   G  Y  Y   T  M  S  N   N  L  V   T  L  E
901    GTGTTACAGT GGGCTGAAAA AGGATACTAC ACCATGAGCA ACAACTTGGT AACCCTGGAA

       N  G  K  Q   L  T  V   K  R  Q   G  L  Y  Y   I  Y  A   Q  V  T
961    AATGGGAAAC AGCTGACCGT TAAAAGACAA GGACTCTATT ATATCTATGC CCAAGTCACC

                  HindIII
                  ~~~~~~~
       F  C  S  N   R  E  A   S  S  Q   A  P  F  I   A  S  L   C  L  K
1021   TTCTGTTCCA ATCGGGAAGC TTCGAGTCAA GCTCCATTTA TAGCCAGCCT CTGCCTAAAG

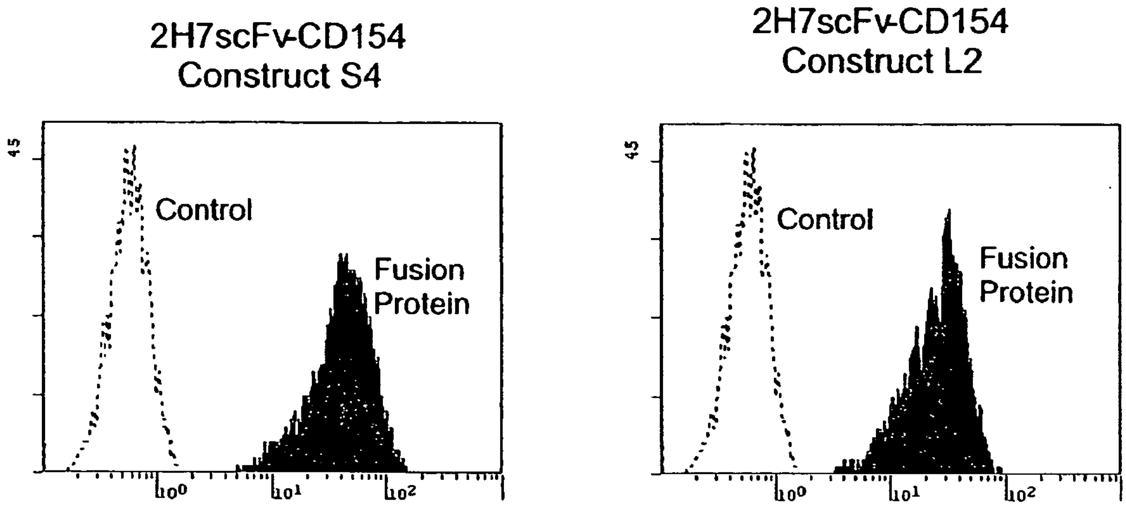
       S  P  G  R   F  E  R   I  L  L   R  A  A  N   T  H  S   S  A  K
1081   TCCCCCGGTA GATTCGAGAG AATCTTACTC AGAGCTGCAA ATACCCACAG TTCCGCCAAA

       P  C  G  Q   Q  S  I   H  L  G   G  V  F  E   L  Q  P   G  A  S
1141   CCTTGCGGGC AACAATCCAT TCACTTGGGA GGAGTATTTG AATTGCAACC AGGTGCTTCG

                                            NcoI
                                          ~~~~~~~
       V  F  V  N   V  T  D   P  S  Q   V  S  H  G   T  G  F   T  S  F
1201   GTGTTTGTCA ATGTGACTGA TCCAAGCCAA GTGAGCCATG GCACTGGCTT CACGTCCTTT

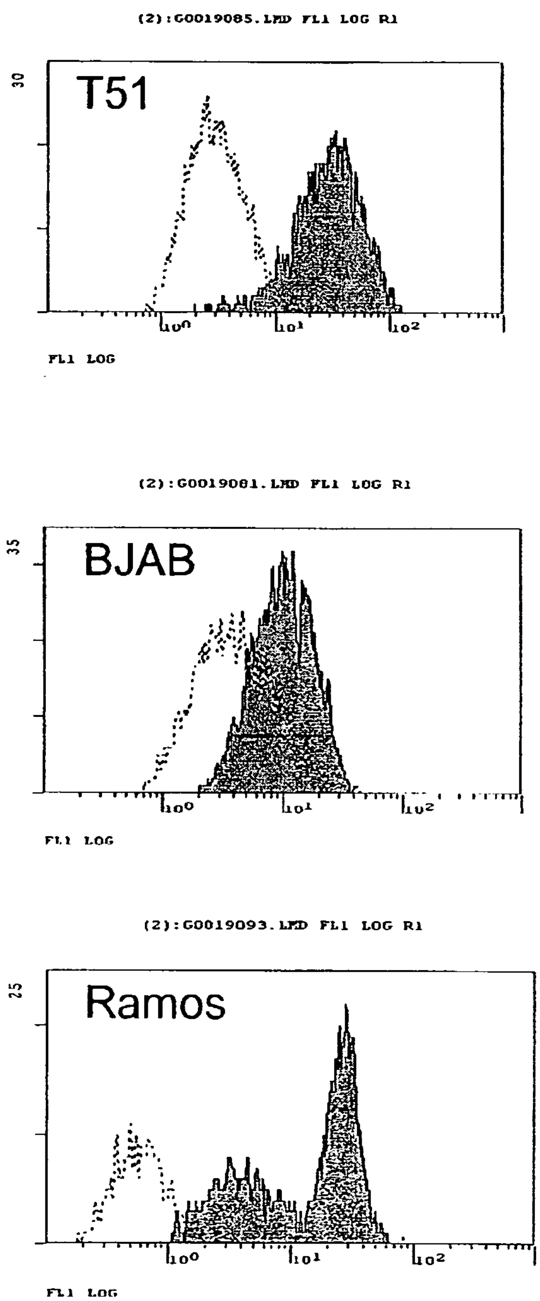
                XhoI      XbaI
               ~~~~~~     ~~~~
       G  L  L  K   L  E  *   *  S  R
1261   GGCTTACTCA AACTCGAGTG ATAATCTAGA
```

Figure 8



CD20 CHO cell targets + (control or fusion protein)
+ Biotin-CD40Ig + PE-SA

Figure 9



......control supernatant ____2H7scFv-CD154 supernatant

Figure 10

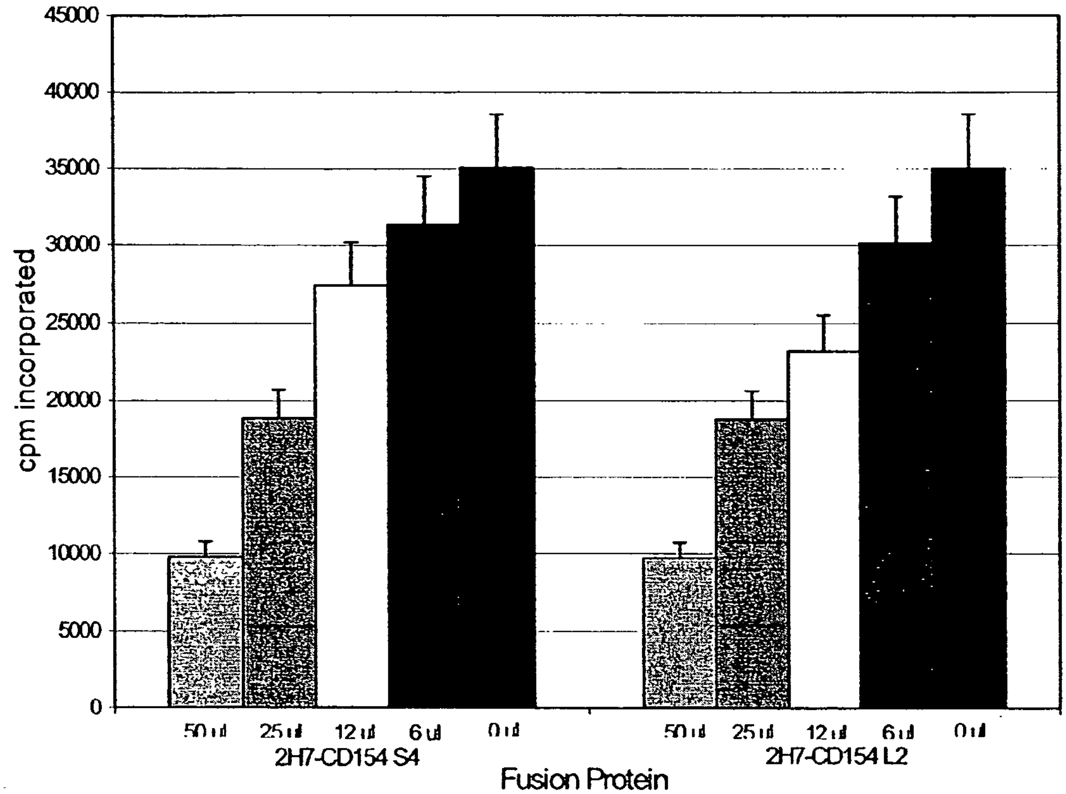

45000
40000
35000
30000
25000
20000
15000
10000
5000
0
cpm incorporated
50 ul
25 ul
12 ul
6 ul
0 ul
2H7-CD154 S4
50 ul
25 ul
12 ul
6 ul
0 ul
2H7-CD154 L2
Fusion Protein

Figure 11

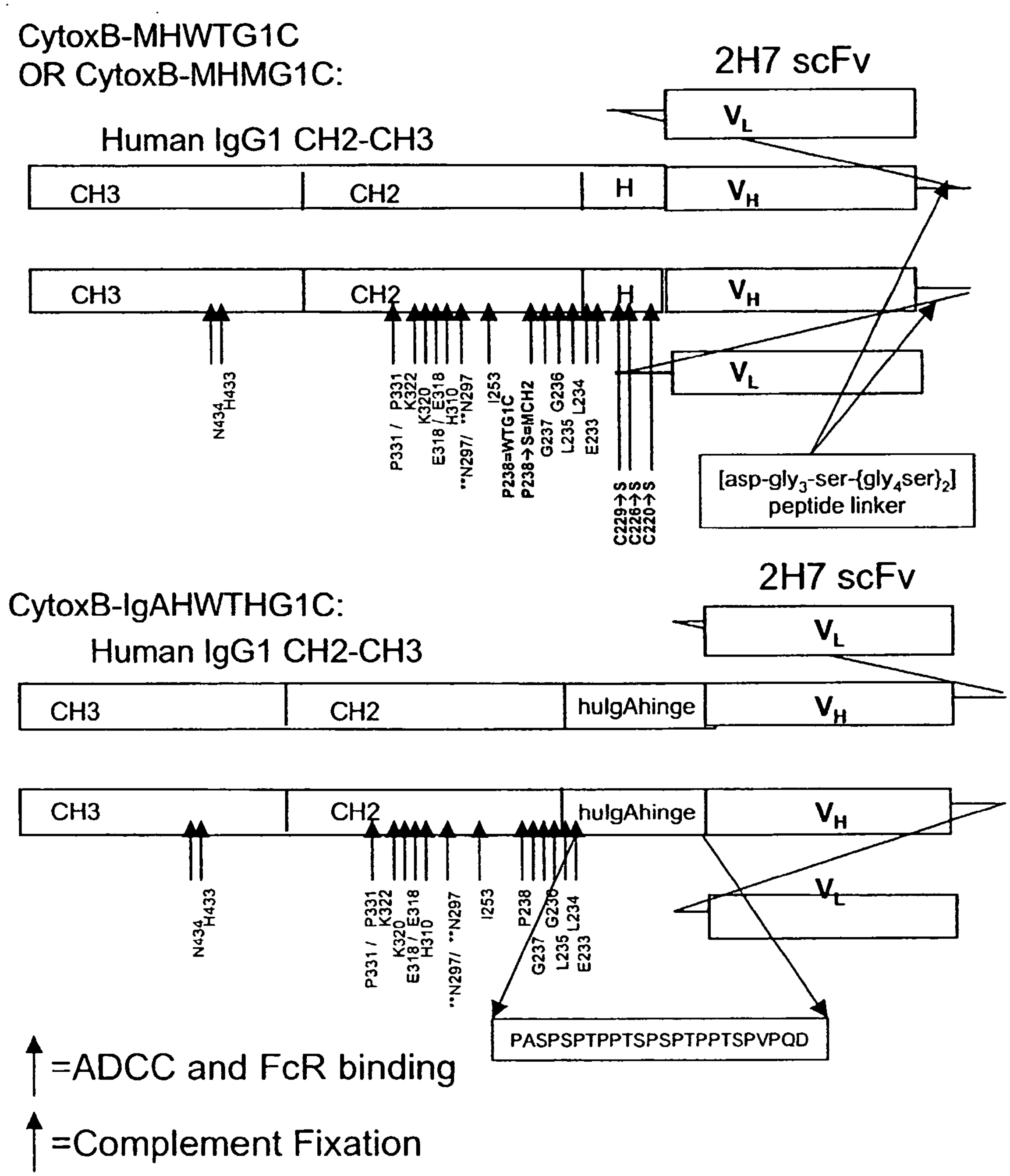

CytoxB-MHWTG1C
OR CytoxB-MHMG1C:
2H7 scFv
Human IgG1 CH2-CH3
CH3
CH2
H
V_L
V_H
N434
H433
P331 / P331
K322
K320
E318 / E318
H310
**N297/ **N297
I253
P238=WTG1C
P238→S=MCH2
G237
G236
L235
L234
E233
C229→S
C226→S
C220→S
[asp-gly$_3$-ser-{gly$_4$ser}$_2$]
peptide linker
CytoxB-IgAHWTHG1C:
Human IgG1 CH2-CH3
2H7 scFv
hulgAhinge
P238
PASPSPTPPTSPSPTPPTSPVPQD
=ADCC and FcR binding
=Complement Fixation

Figure 12

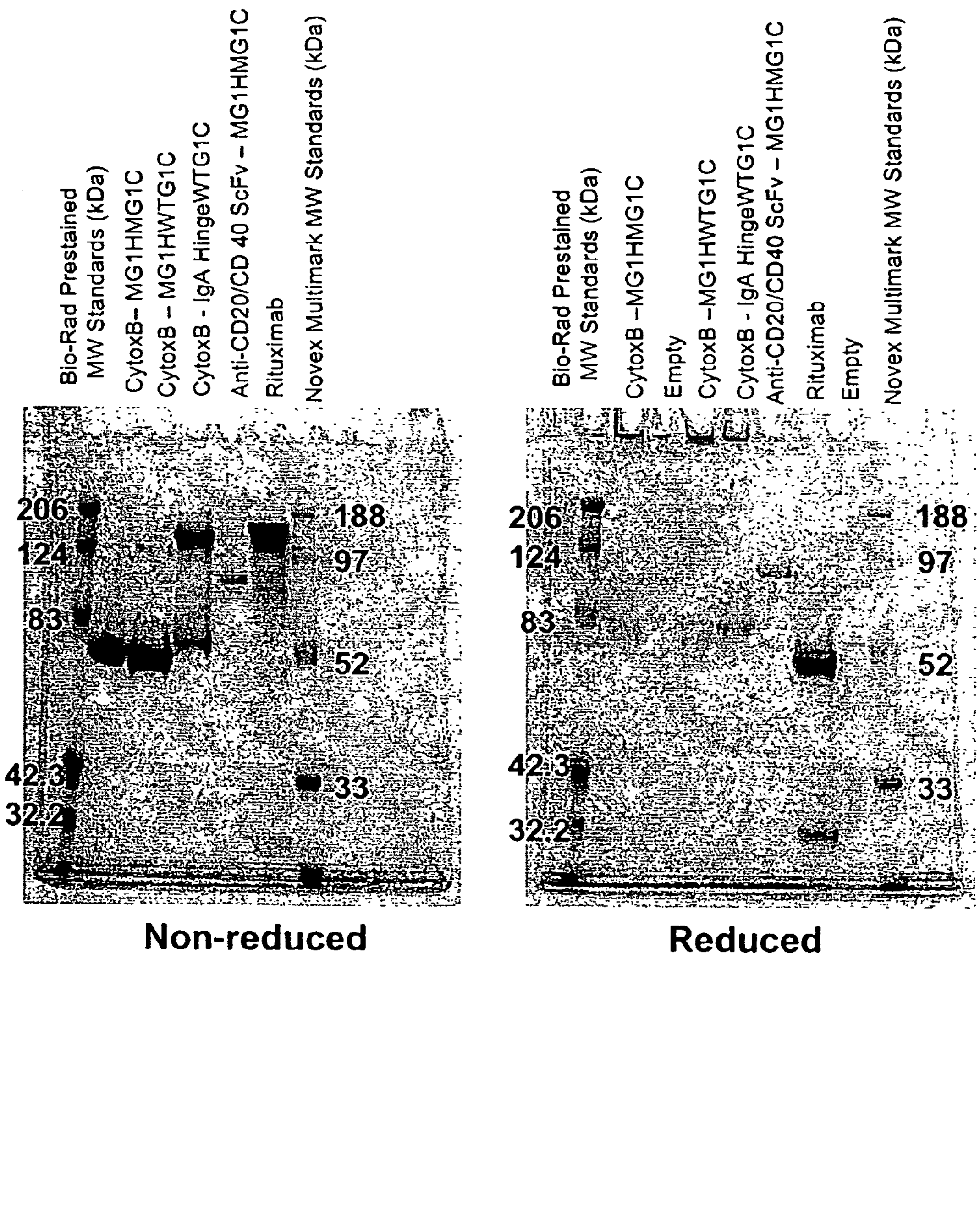

Bio-Rad Prestained MW Standards (kDa)
CytoxB – MG1HMG1C
CytoxB – MG1HWTG1C
CytoxB - IgA HingeWTG1C
Anti-CD20/CD 40 ScFv – MG1HMG1C
Rituximab
Novex Multimark MW Standards (kDa)
206
124
83
42.3
32.2
188
97
52
33
Non-reduced
Bio-Rad Prestained MW Standards (kDa)
CytoxB –MG1HMG1C
Empty
CytoxB –MG1HWTG1C
CytoxB - IgA HingeWTG1C
Anti-CD20/CD40 ScFv – MG1HMG1C
Rituximab
Empty
Novex Multimark MW Standards (kDa)
206
124
83
42.3
32.2
188
97
52
33
Reduced

Figure 13

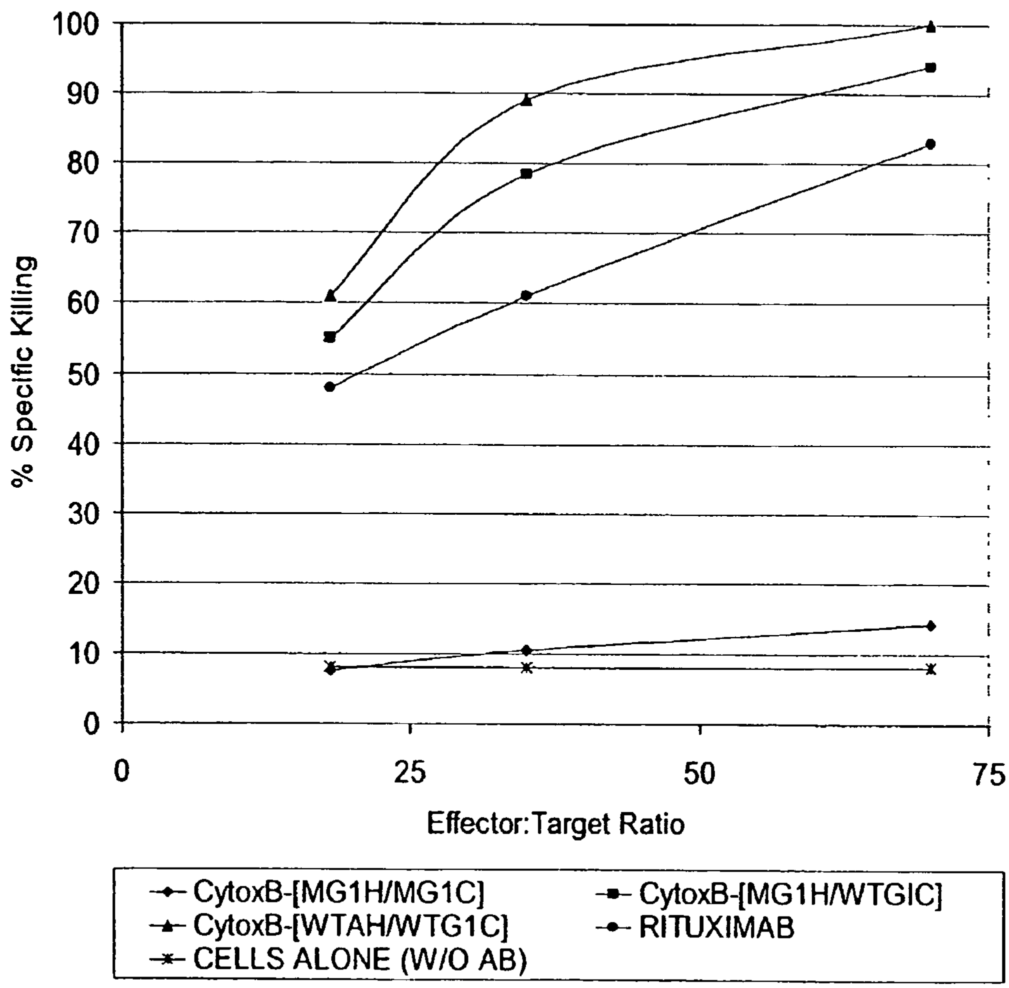

100
90
80
70
60
50
40
30
20
10
0
% Specific Killing
0
25
50
75
Effector:Target Ratio
CytoxB-[MG1H/MG1C]
CytoxB-[MG1H/WTGIC]
CytoxB-[WTAH/WTG1C]
RITUXIMAB
CELLS ALONE (W/O AB)

Figure 14

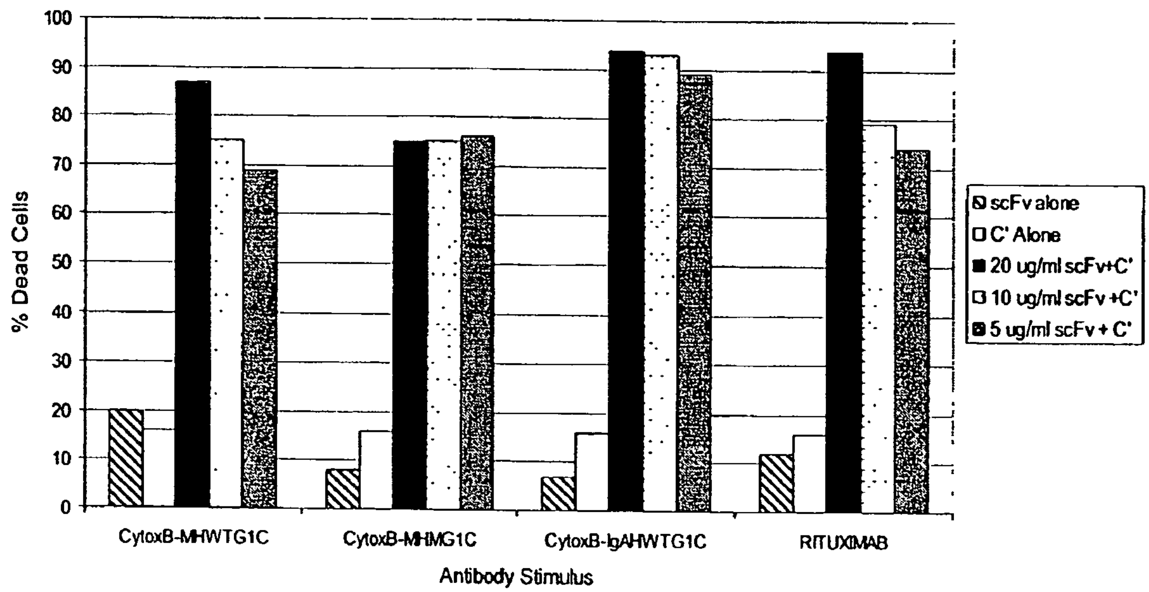
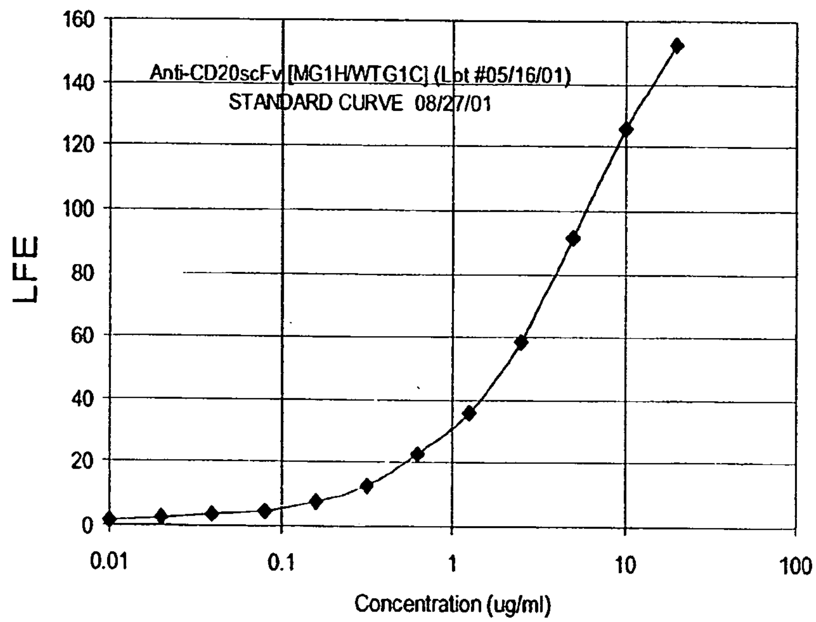

100
90
80
70
60
50
40
30
20
10
0
% Dead Cells
CytoxB-MHWTG1C
CytoxB-MHMG1C
CytoxB-IgAHWTG1C
RITUXIMAB
Antibody Stimulus
scFv alone
C' Alone
20 ug/ml scFv+C'
10 ug/ml scFv +C'
5 ug/ml scFv + C'

Figure 15



	Day	Monkey J99231 LFE(1:40)	Monkey J99231 Concentration (μg/mL)	Monkey K99334 LFE(1:40)	Monkey K99334 Concentration (μg/mL)
Injection	-7	2.41	<0.6μg/mL	1.51	<0.4μg/mL
	0	2.22	<0.6μg/mL	1.63	<0.4μg/mL
	1	73.8	220μg/mL	44.4	100μg/mL
Injection	3	20.0	28μg/mL	40.2	80μg/mL
	7	15.6	24μg/mL	15.7	24μg/mL
	8	39.1	80μg/mL	42.6	92μg/mL
	10	11.5	18μg/mL	2.74	1.2μg/mL
	14	2.05	0.6mg/mL	1.96	0.6μg/mL

Figure 16

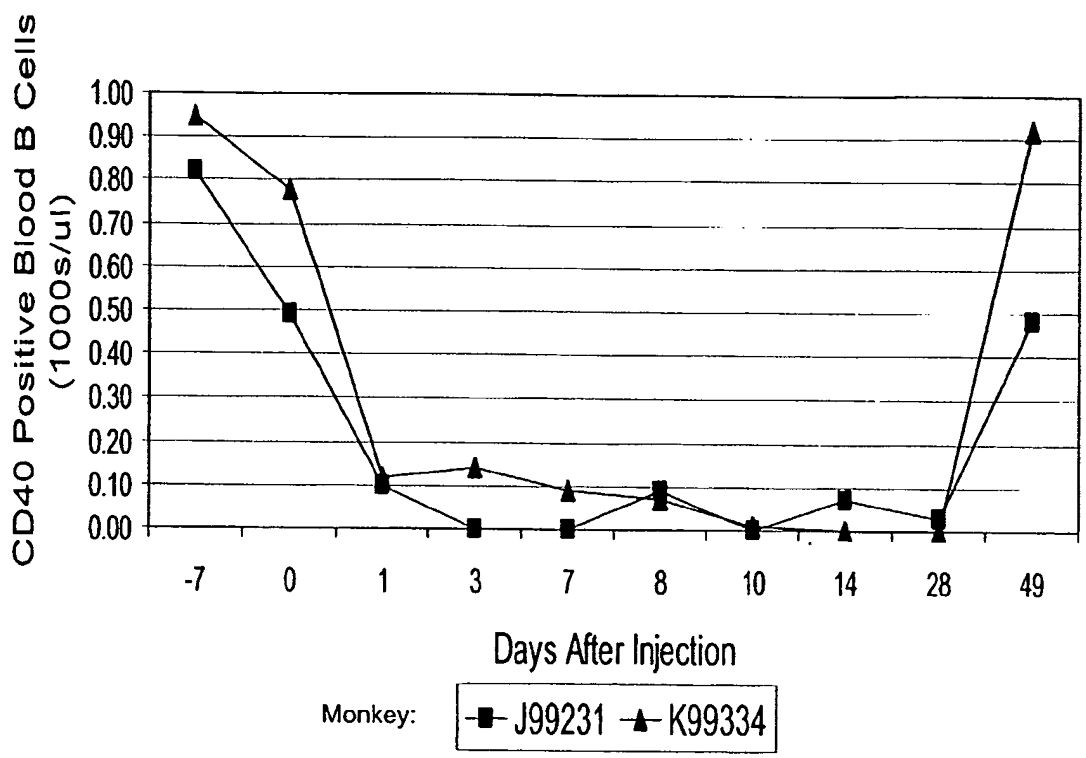
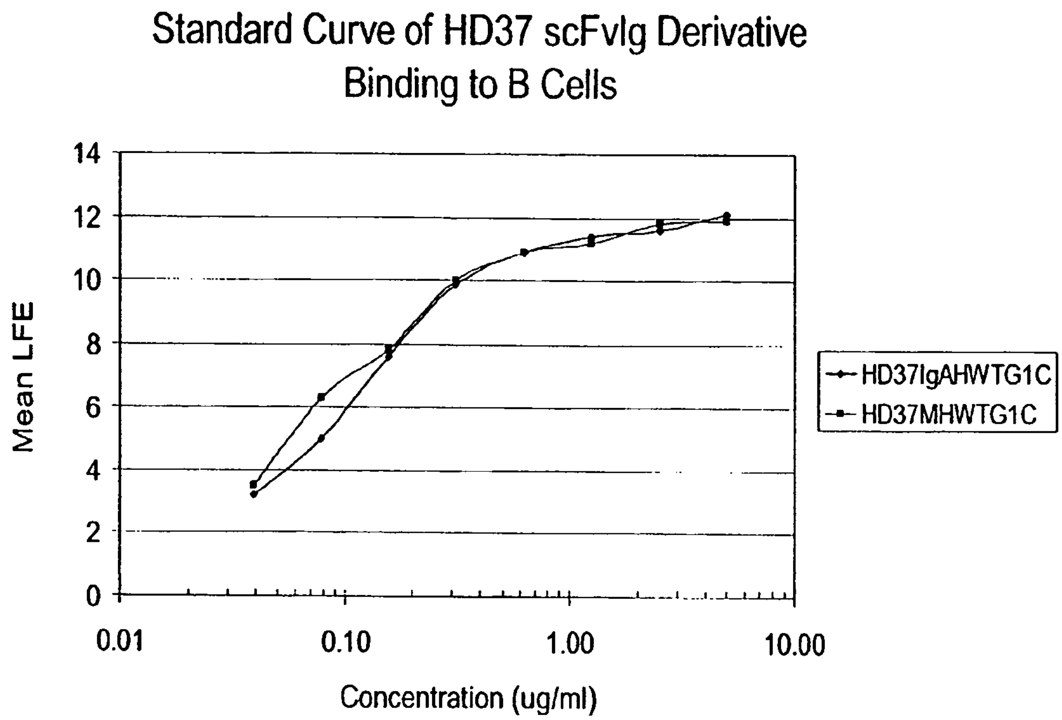
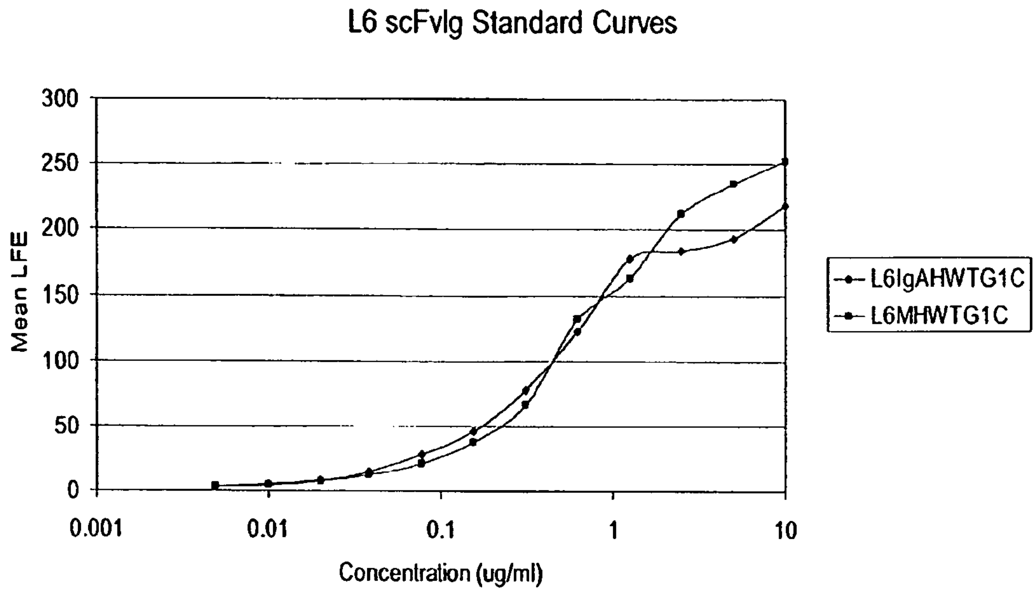

CD40 Positive Blood B Cells
(1000s/ul)
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
-7
0
1
3
7
8
10
14
28
49
Days After Injection
Monkey:
J99231
K99334

Figure 17



Clone/Isolate	Mean LFE at 1:100	Estimated Concentration
Bulk IgAHWTG1C	11.2	> 60 ug/ml
1B2	10.4	>50 ug/ml
6C5	10.5	>50 ug/ml
4B1	8.6	>40 ug/ml
Bulk MHWTG1C	10.9	> 50 ug/ml
2G8	10.6	> 50 ug/ml
3F3	8.3	>40 ug/ml
3D9	11.1	> 60 ug/ml

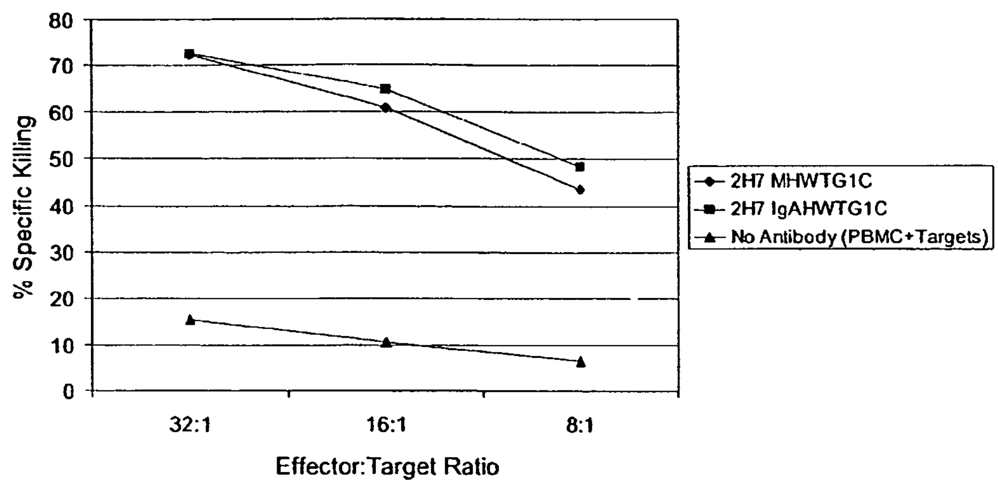
Figure 18



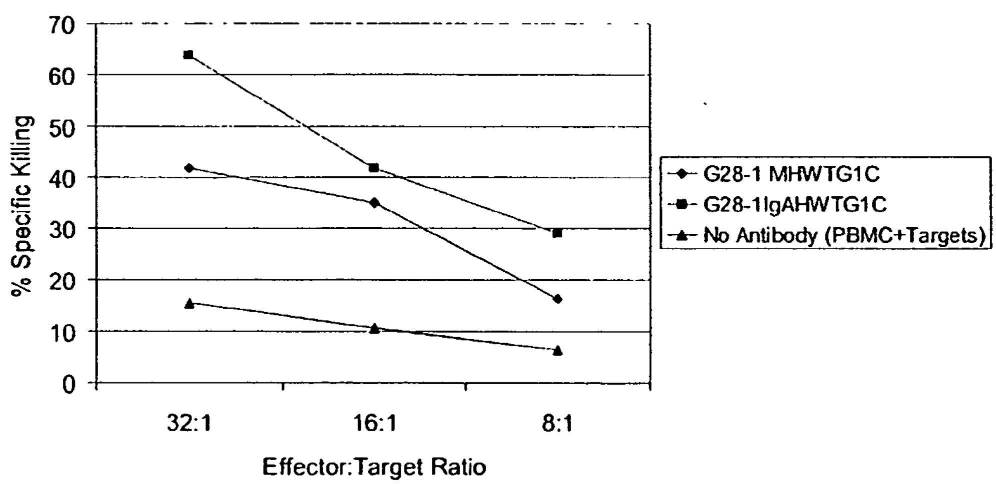
Construct	Mean LFE 1:20	Estimated Concentration
L6IgAHWTG1C unamplified CHO sup	51.1	6.25 ug/ml
L6IgGMHWTG1C unamplified CHO sup	23.0	3.2 ug/ml

Figure 19

A. 2H7 (anti-CD20) scFv Derivatives



B. G28-1 (anti-CD37) scFv Derivatives



C. HD37 (anti-CD19) scFv Derivatives

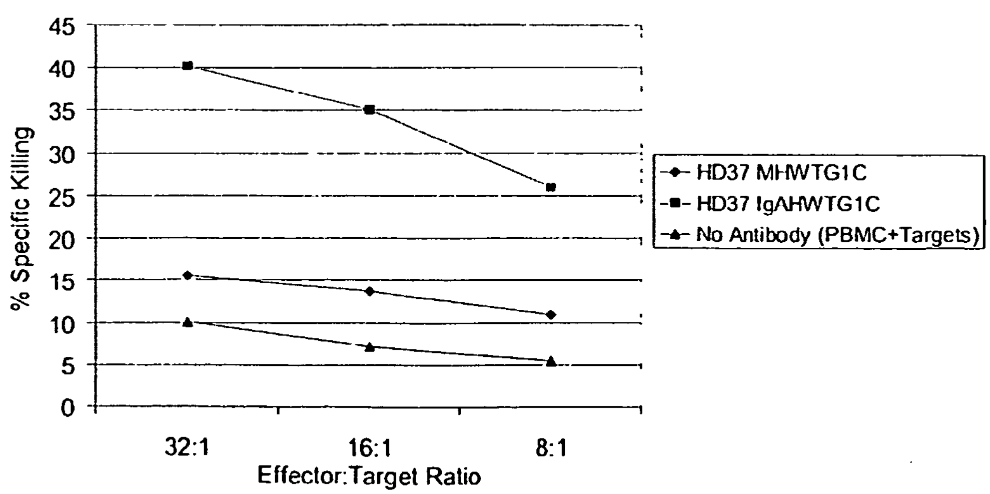


Figure 20

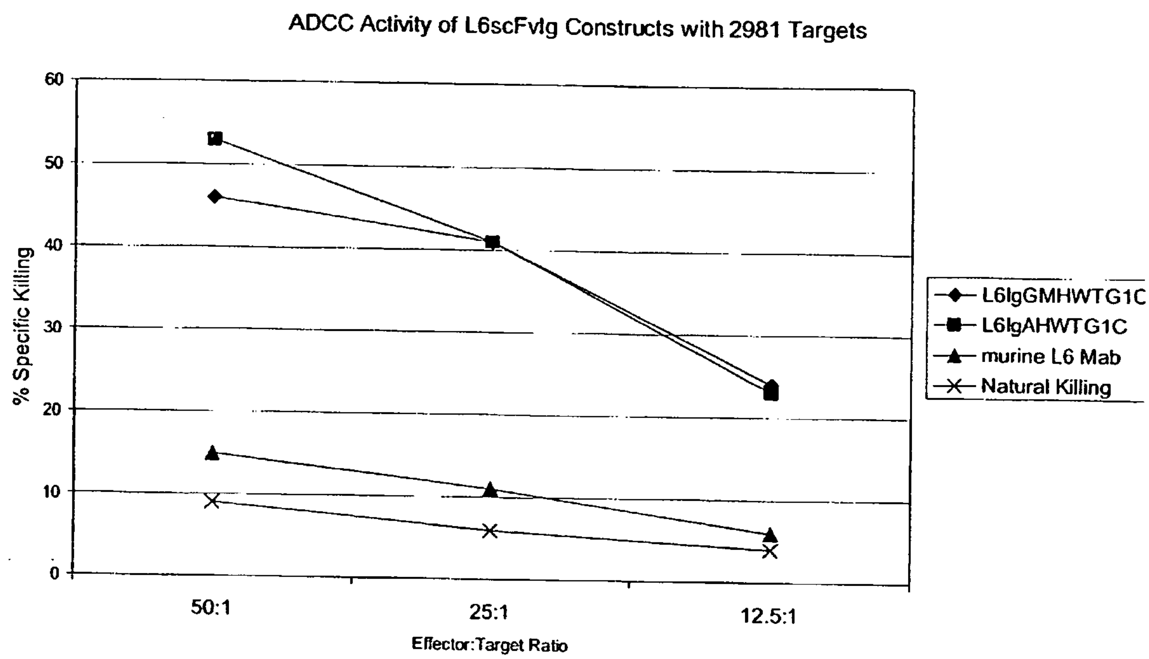

ADCC Activity of L6scFvIg Constructs with 2981 Targets
60
50
40
30
20
10
0
% Specific Killing
50:1
25:1
12.5:1
Effector:Target Ratio
L6IgGMHWTG1C
L6IgAHWTG1C
murine L6 Mab
Natural Killing

Figure 21

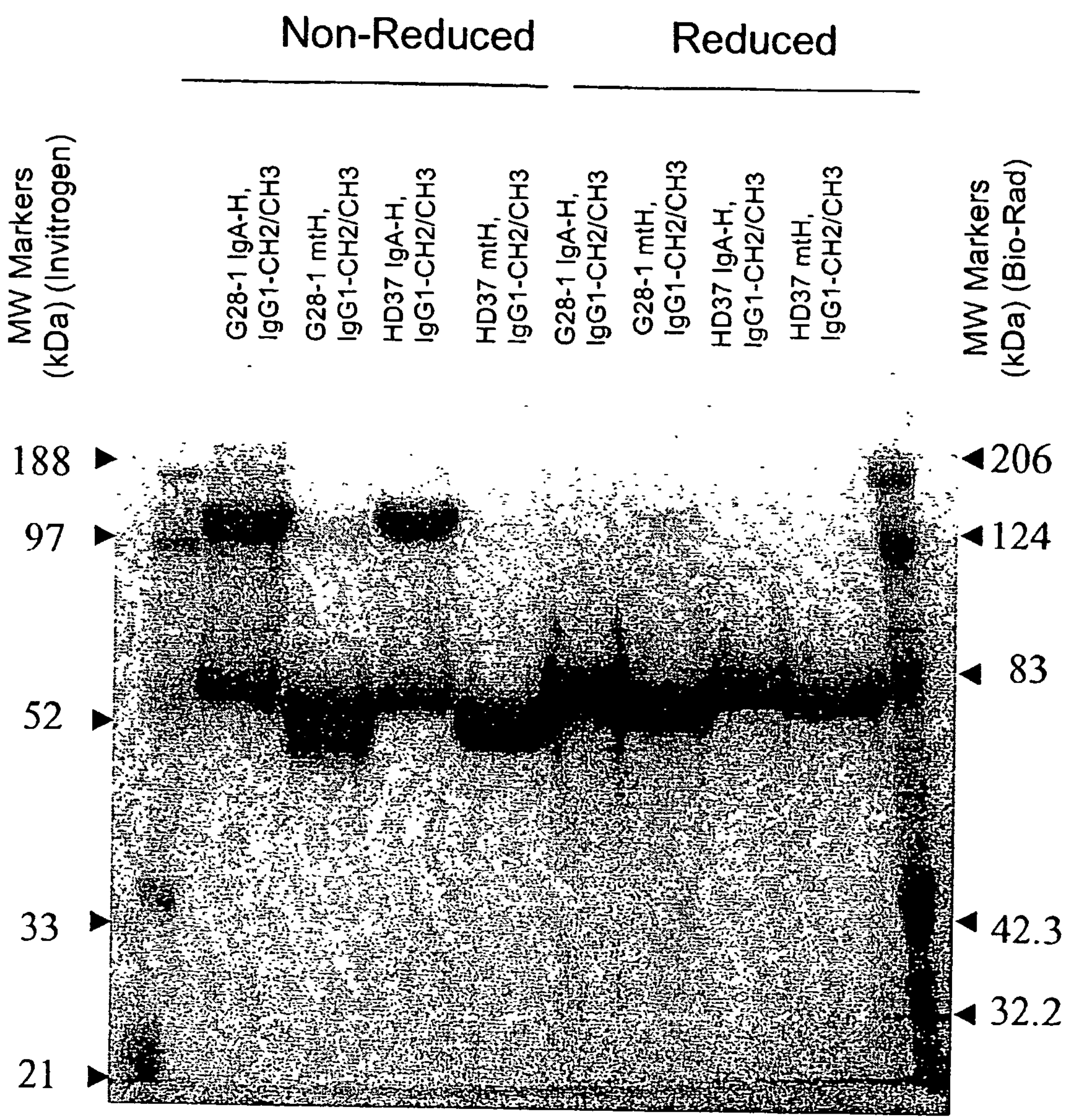
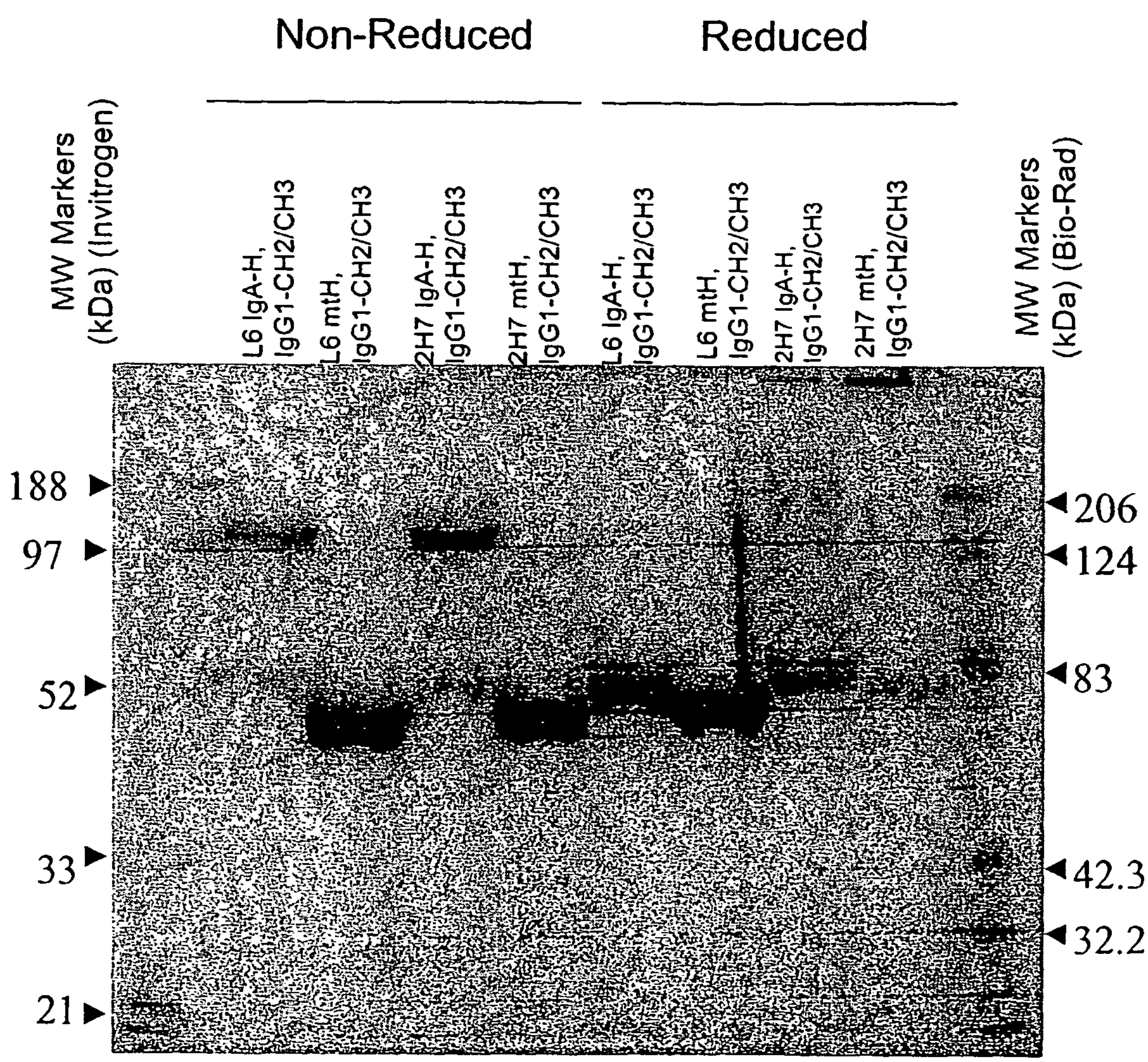

Non-Reduced
Reduced
MW Markers (kDa) (Invitrogen)
G28-1 IgA-H, IgG1-CH2/CH3
G28-1 mtH, IgG1-CH2/CH3
HD37 IgA-H, IgG1-CH2/CH3
HD37 mtH, IgG1-CH2/CH3
G28-1 IgA-H, IgG1-CH2/CH3
G28-1 mtH, IgG1-CH2/CH3
HD37 IgA-H, IgG1-CH2/CH3
HD37 mtH, IgG1-CH2/CH3
MW Markers (kDa) (Bio-Rad)
188
97
52
33
21
206
124
83
42.3
32.2

Figure 22



Non-Reduced
Reduced
MW Markers (kDa) (Invitrogen)
L6 IgA-H, IgG1-CH2/CH3
L6 mtH, IgG1-CH2/CH3
2H7 IgA-H, IgG1-CH2/CH3
2H7 mtH, IgG1-CH2/CH3
L6 IgA-H, IgG1-CH2/CH3
L6 mtH, IgG1-CH2/CH3
2H7 IgA-H, IgG1-CH2/CH3
2H7 mtH, IgG1-CH2/CH3
MW Markers (kDa) (Bio-Rad)
188
97
52
33
21
206
124
83
42.3
32.2

Figure 23

```
                          Hinge                                          CH2
Human IgG1: DQEPKSCDKT------------HTCPPC PAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDG
Llama IgG2: DQEPKTPKPQPQPQPQPNPTTESKCPKC PAPELLGGPSVFIFPPKPKDVLSISGRPEVTCVVVDVGQEDPEVSFNWYIDG
Llama IgG1: --EPHGG---------------CTCPQC PAPELPGGPSVFVFPPKPKDVLSISGRPEVTCVVVDVGKEDPEVNFNWYIDG
Llama IgG3: --AHHSEDPT------------SKCPKC PGPELLGGPTVFIFPPKAKDVLSITRKPEVTCLWWTWVKKTLRSSSSWSVDD

                                                                      CH3
VEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLT
TAEVRANTRPKEEQFNSTYRVVSVLPIQHQDWLTGKEFKCKVNNKALPAPIEKTISKAKGQTREPQVYTLAPHREELAKDTVSVT
VEVRTANTKPKEEQFNSTYRVVSVLPIQHQDWLTGKEFKCKVNNKALPAPIERTISKAKGQTREPQVYTLAPHREELAKDTVSVT
TEVHTAETKPKEEQFNSTYRVVSVLPIQHQDWLTGKEFKCKVNNKALPAPIERTISKAKGQTREPQVYTLAPHREELAKDTVSVT

CLVKGFYPSDIAVEWESNGQPEN--NYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK
CLVKGFYPPDINVEWQRNGQPESXGTYATTPPQLDNDGTYFLXSKXSVGKNTWQQGETFTCVVMHEALHNHYTQKSITQSSGK
CLVKGFYPADINVEWQRNGQPESEGTYANTPPQLDNDGTYFLYSRLSVGKNTWQRGETLTGVVMHEALHNHYTQKSITQSSGK
CLVKGFFPADINVEWQRNGQPESEGTYANTPPQLDNDGTYFLYSKLSVGKNTWQQGEVFTCVVMHEALHNHSTQKSITQSSGK
```

Figure 24

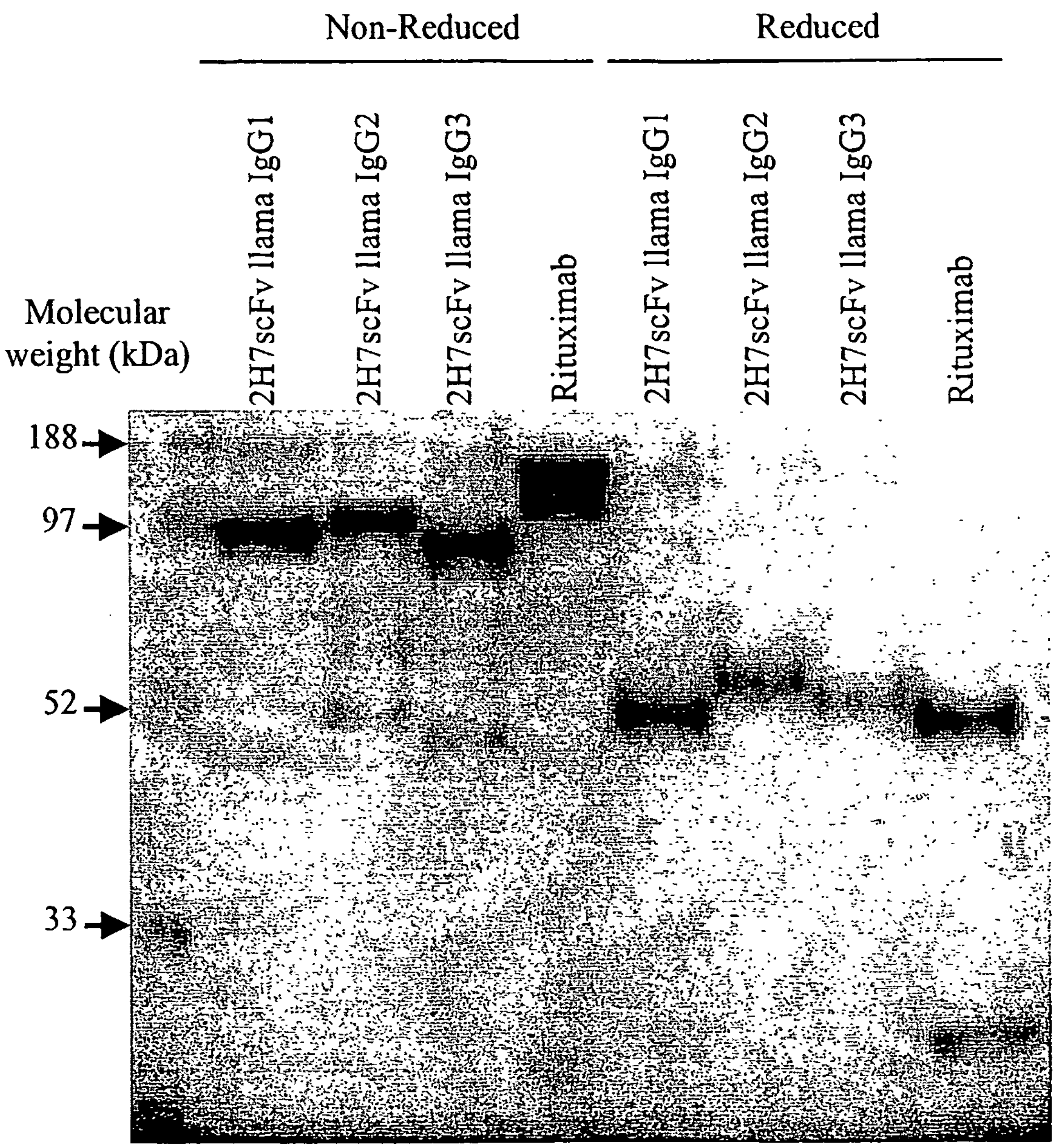

Non-Reduced
Reduced
2H7scFv llama IgG1
2H7scFv llama IgG2
2H7scFv llama IgG3
Rituximab
2H7scFv llama IgG1
2H7scFv llama IgG2
2H7scFv llama IgG3
Rituximab
Molecular weight (kDa)
188
97
52
33

Figure 25

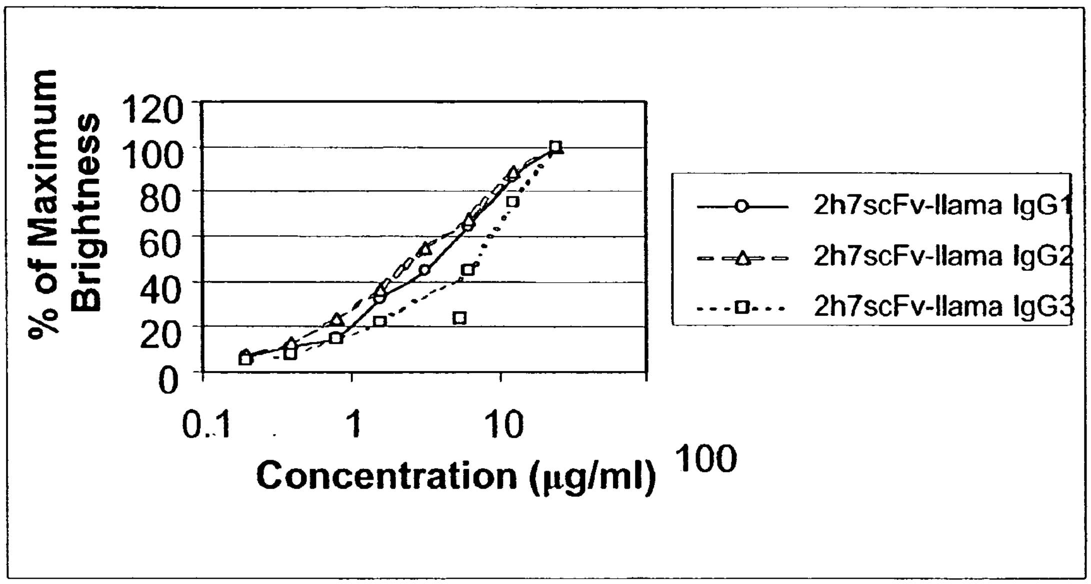

% of Maximum Brightness
120
100
80
60
40
20
0
0.1
1
10
100
Concentration (µg/ml)
2h7scFv-llama IgG1
2h7scFv-llama IgG2
2h7scFv-llama IgG3

Figure 26

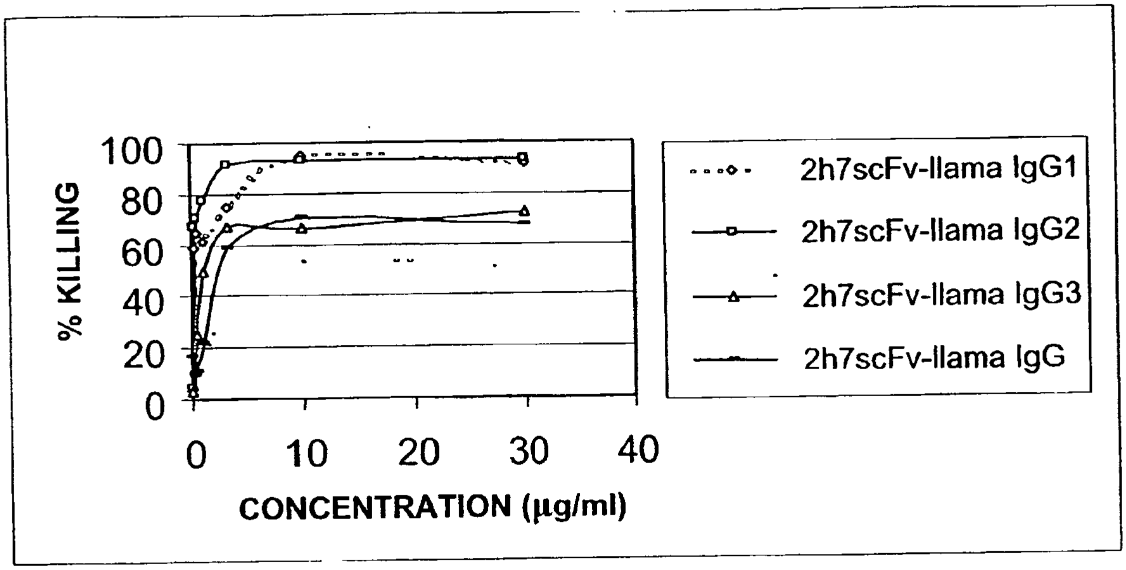

% KILLING
100
80
60
40
20
0
0
10
20
30
40
CONCENTRATION (μg/ml)
2h7scFv-llama IgG1
2h7scFv-llama IgG2
2h7scFv-llama IgG3
2h7scFv-llama IgG

Figure 27

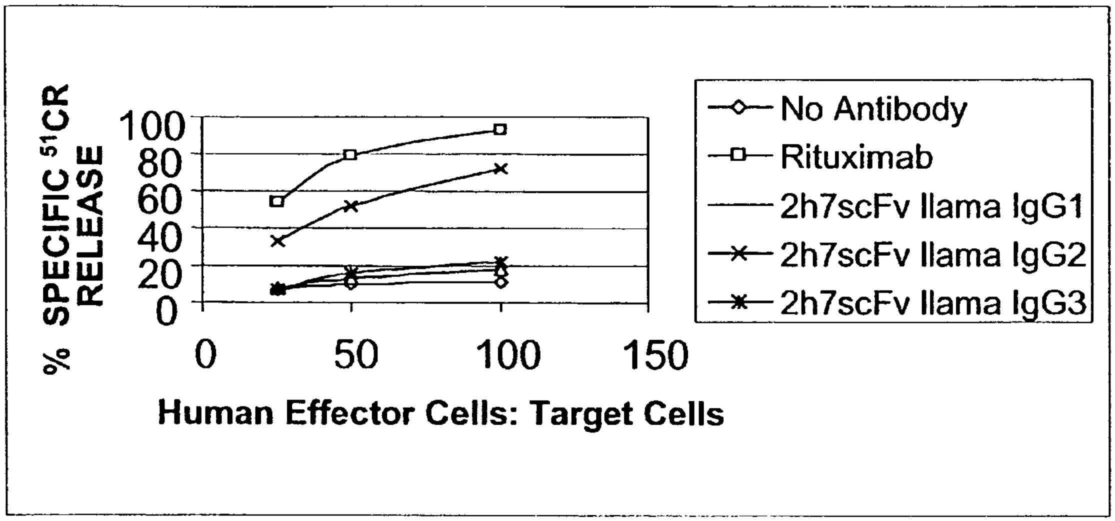

% SPECIFIC 51CR RELEASE
100
80
60
40
20
0
0
50
100
150
Human Effector Cells: Target Cells
No Antibody
Rituximab
2h7scFv llama IgG1
2h7scFv llama IgG2
2h7scFv llama IgG3

Figure 28

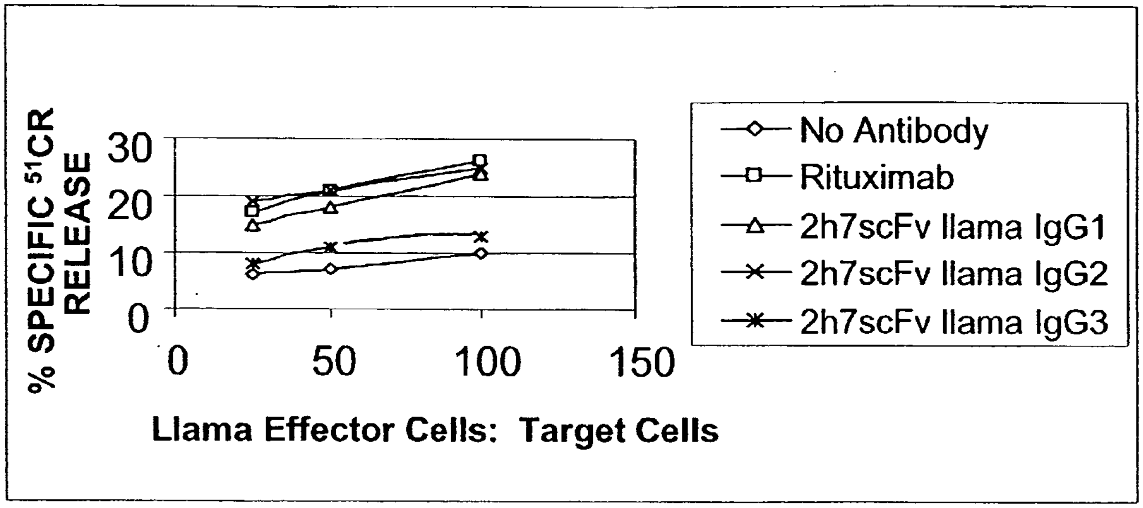

% SPECIFIC 51CR RELEASE
30
20
10
0
0
50
100
150
Llama Effector Cells: Target Cells
No Antibody
Rituximab
2h7scFv llama IgG1
2h7scFv llama IgG2
2h7scFv llama IgG3

Figure 29

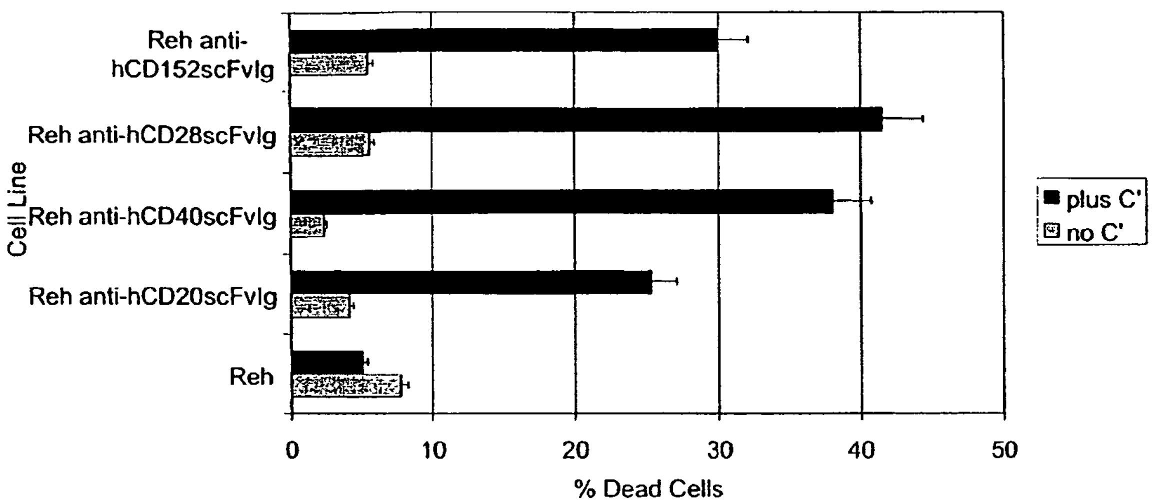
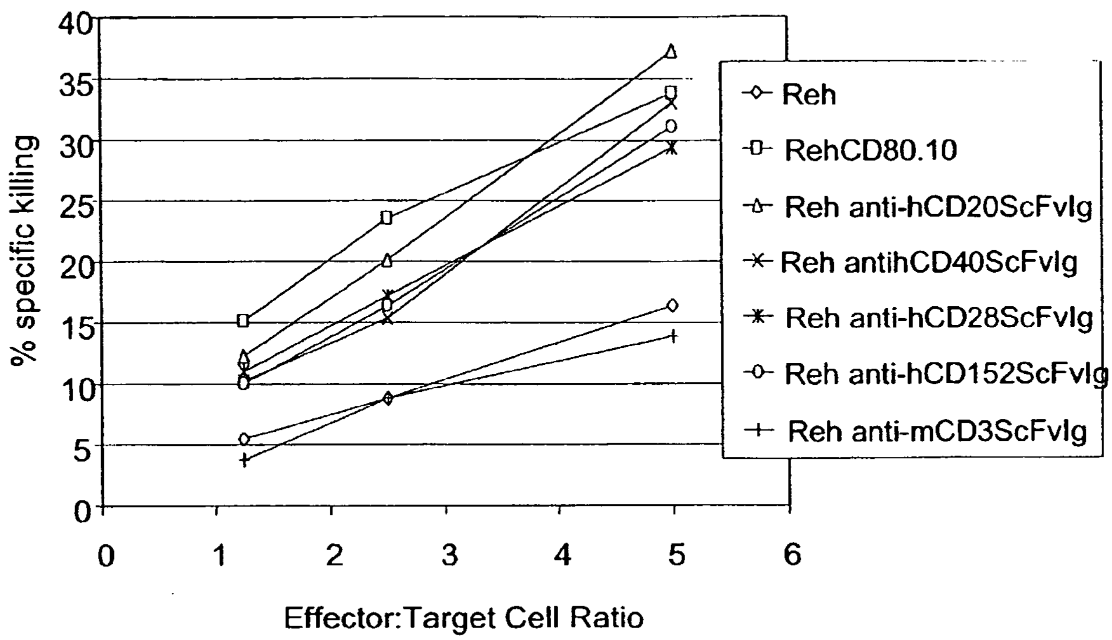

Reh anti-hCD152scFvIg
Reh anti-hCD28scFvIg
Reh anti-hCD40scFvIg
Reh anti-hCD20scFvIg
Reh
Cell Line
0
10
20
30
40
50
% Dead Cells
plus C'
no C'

Figure 30



40
35
30
25
20
15
10
5
0
% specific killing
0
1
2
3
4
5
6
Effector:Target Cell Ratio
Reh
RehCD80.10
Reh anti-hCD20ScFvIg
Reh antihCD40ScFvIg
Reh anti-hCD28ScFvIg
Reh anti-hCD152ScFvIg
Reh anti-mCD3ScFvIg

Figure 31

Name Identifier	Hinge Sequence	CH2 Sequence	CH3 Sequence	SEQ ID NO:
IgG WTH (CCC) WTCH2CH3	IgG WT Hinge (CCC)	Wild Type CH2	Wild Type CH3	
IgG MTH (SSS) WTCH2CH3	IgG1 Mutant Hinge (SSS)	Wild type CH2 (IgG1)	Wild type CH3 (IgG1)	
VH SER 11 IgG MTH (SSS) WTCH2CH3	IgG1 Mutant Hinge (SSS)	Wild type CH2 (IgG1)	Wild type CH3 (IgG1)	
IgG (SSC) WTCH2CH3	IgG1 Mutant Hinge (SSC)	Wild type CH2 (IgG1)	Wild type CH3 (IgG1)	
IgG (SCS) WTCH2CH3	IgG1 Mutant Hinge (SCS)	Wild type CH2 (IgG1)	Wild type CH3 (IgG1)	
IgG (CSS) WTCH2CH3	IgG1 Mutant Hinge (CSS)	Wild type CH2 (IgG1)	Wild type CH3 (IgG1)	
IgG MTH (SSS) MTCH2WTCH3	IgG1 Mutant Hinge (SSS)	Mutant CH2 (IgG1) Pro → Ser 238	Wild type CH3 (IgG1)	
IgAH IgGWTCH2CH3	IgA Hinge	Wild type IgG1 CH2	Wild type IgG1 CH3	
IgAH IgACH2CH3	IgA Hinge	Wild type CH2 (IgA)	Wild type CH3 (IgA)	
IgAH IgA-T4	IgA Hinge	Wild type CH2 (IgA)	Truncated CH3 (IgA) (deletion of 4 amino acids at carboxy terminus)	

Figure 32

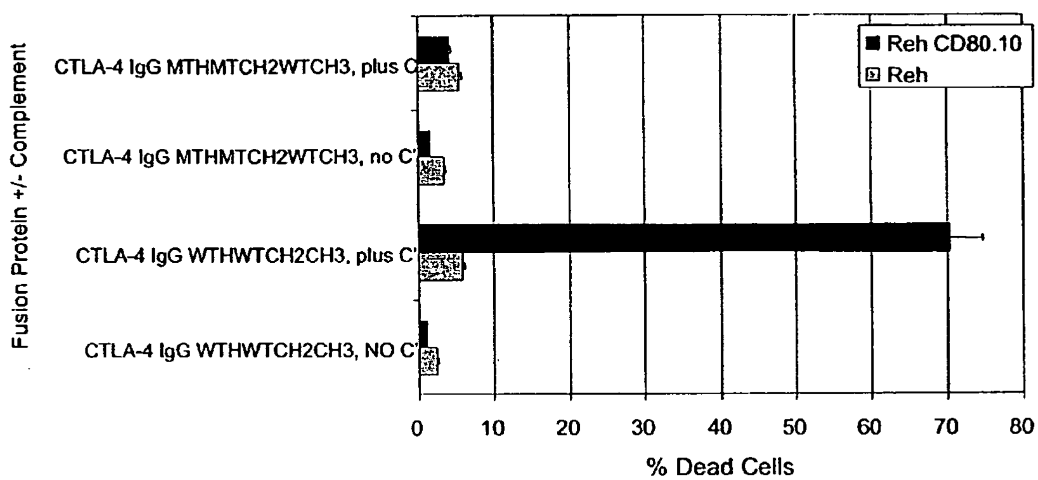

Fusion Protein +/- Complement
CTLA-4 IgG MTHMTCH2WTCH3, plus C'
CTLA-4 IgG MTHMTCH2WTCH3, no C'
CTLA-4 IgG WTHWTCH2CH3, plus C'
CTLA-4 IgG WTHWTCH2CH3, NO C'
Reh CD80.10
Reh
0
10
20
30
40
50
60
70
80
% Dead Cells

Figure 33

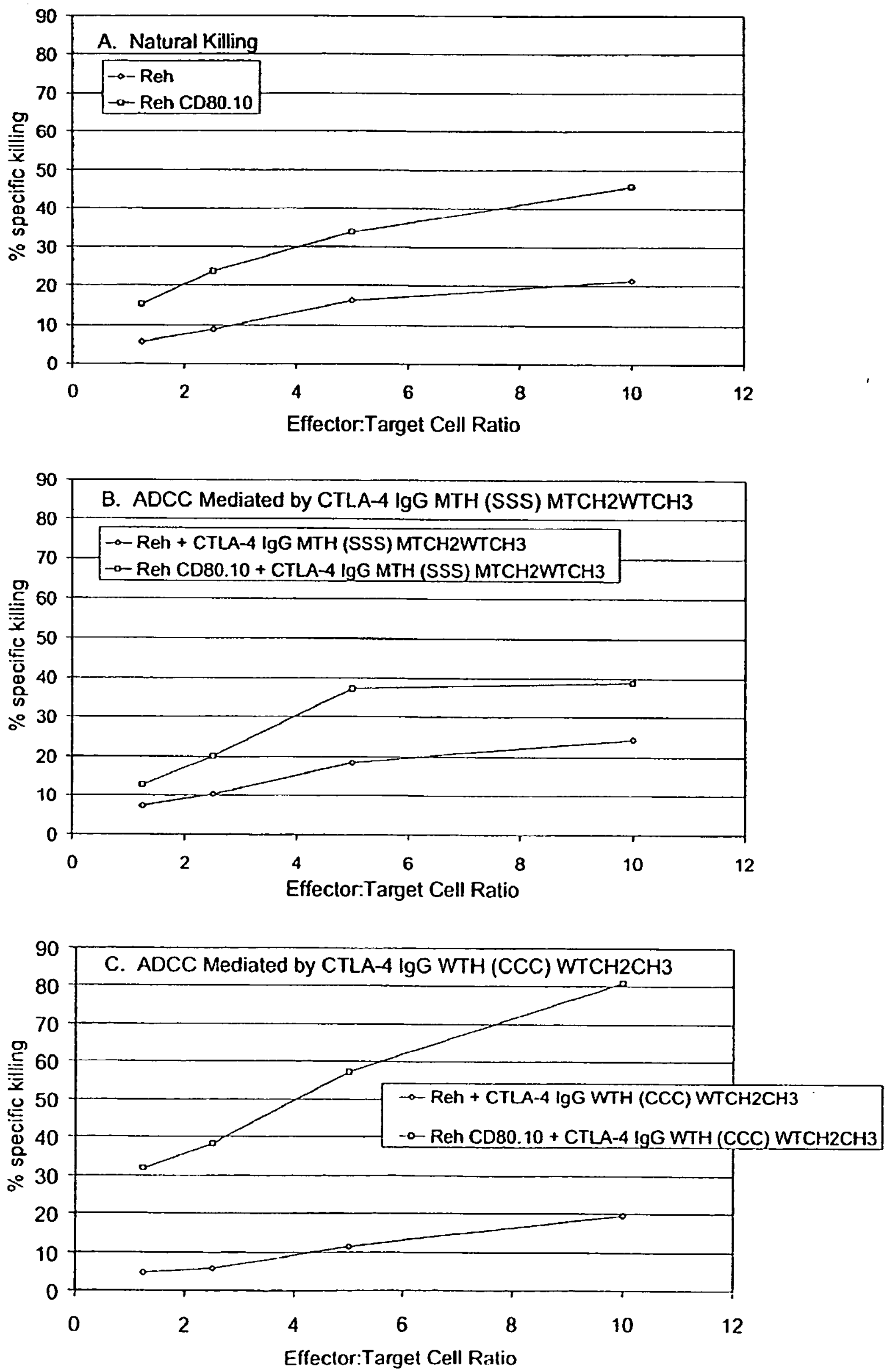

A. Natural Killing
Reh
Reh CD80.10
% specific killing
Effector:Target Cell Ratio
B. ADCC Mediated by CTLA-4 IgG MTH (SSS) MTCH2WTCH3
Reh + CTLA-4 IgG MTH (SSS) MTCH2WTCH3
Reh CD80.10 + CTLA-4 IgG MTH (SSS) MTCH2WTCH3
% specific killing
Effector:Target Cell Ratio
C. ADCC Mediated by CTLA-4 IgG WTH (CCC) WTCH2CH3
Reh + CTLA-4 IgG WTH (CCC) WTCH2CH3
Reh CD80.10 + CTLA-4 IgG WTH (CCC) WTCH2CH3
% specific killing
Effector:Target Cell Ratio

Figure 34

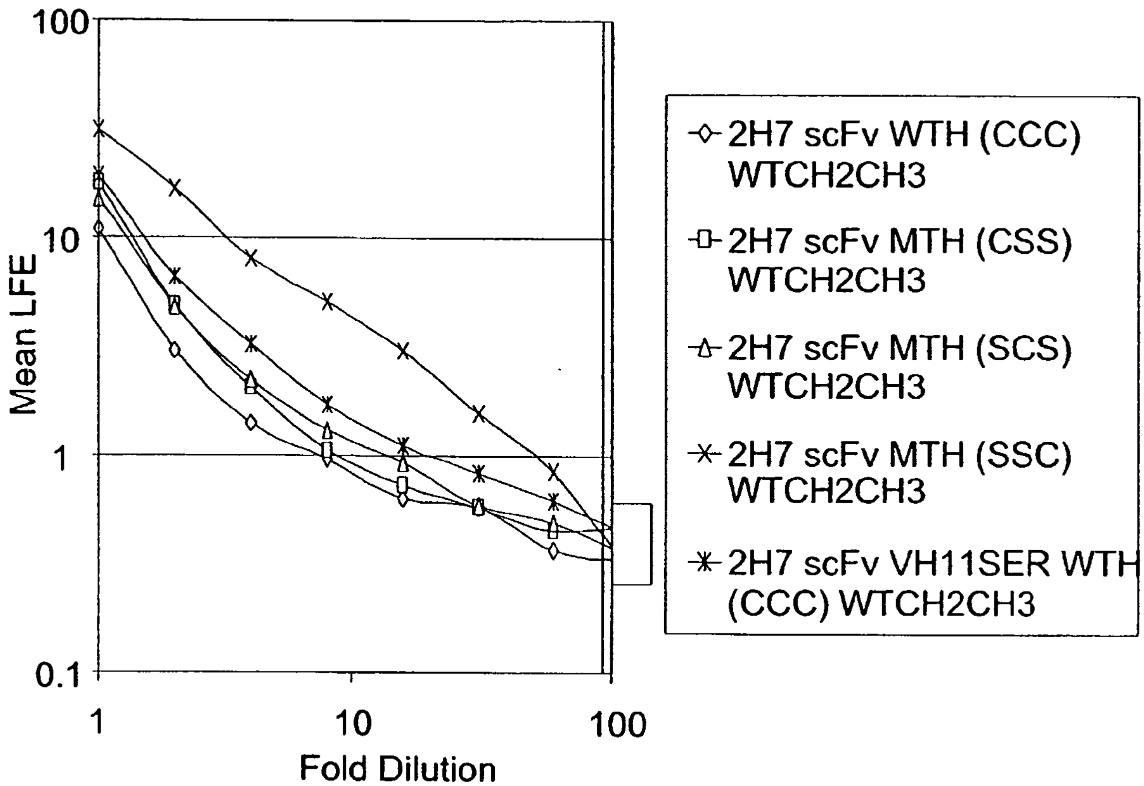

100
10
1
0.1
Mean LFE
1
10
100
Fold Dilution
2H7 scFv WTH (CCC) WTCH2CH3
2H7 scFv MTH (CSS) WTCH2CH3
2H7 scFv MTH (SCS) WTCH2CH3
2H7 scFv MTH (SSC) WTCH2CH3
2H7 scFv VH11SER WTH (CCC) WTCH2CH3

Figure 35

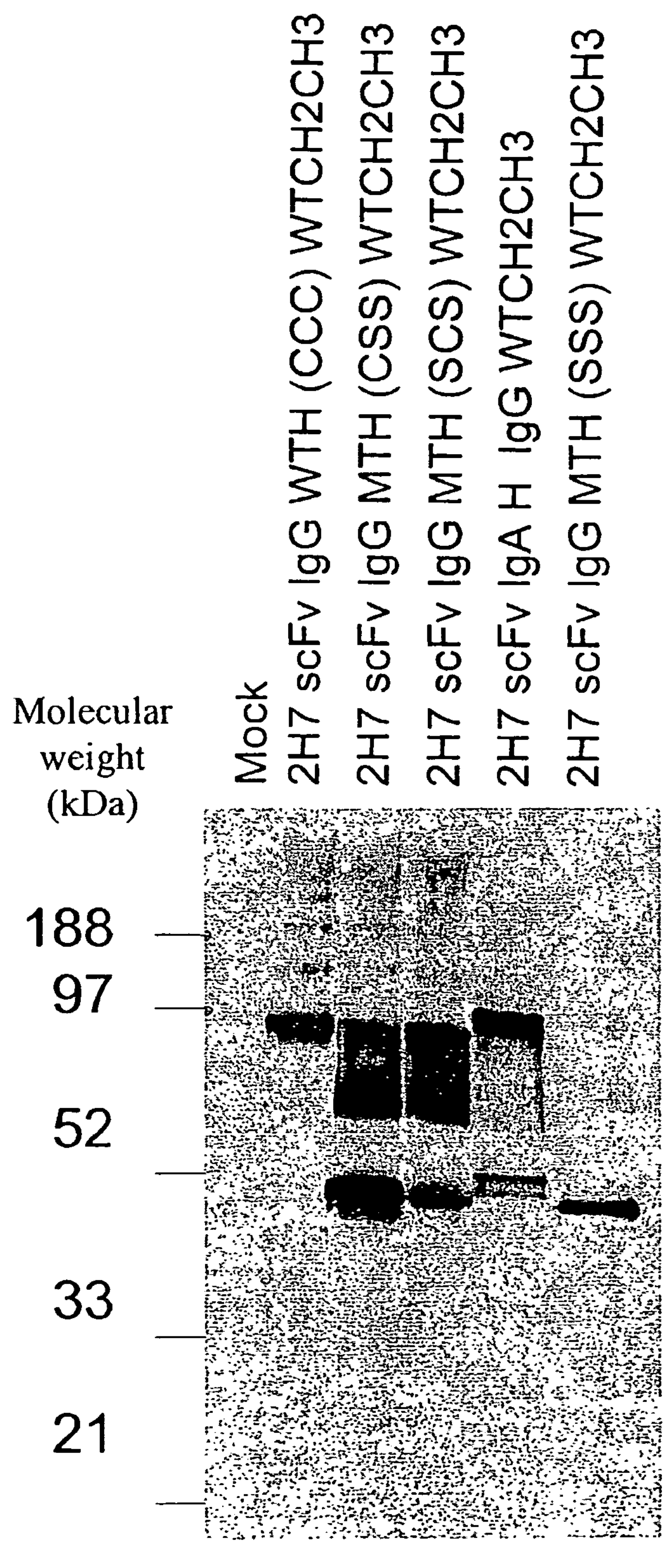

Molecular weight (kDa)
Mock
2H7 scFv IgG WTH (CCC) WTCH2CH3
2H7 scFv IgG MTH (CSS) WTCH2CH3
2H7 scFv IgG MTH (SCS) WTCH2CH3
2H7 scFv IgA H IgG WTCH2CH3
2H7 scFv IgG MTH (SSS) WTCH2CH3
188
97
52
33
21

Figure 36

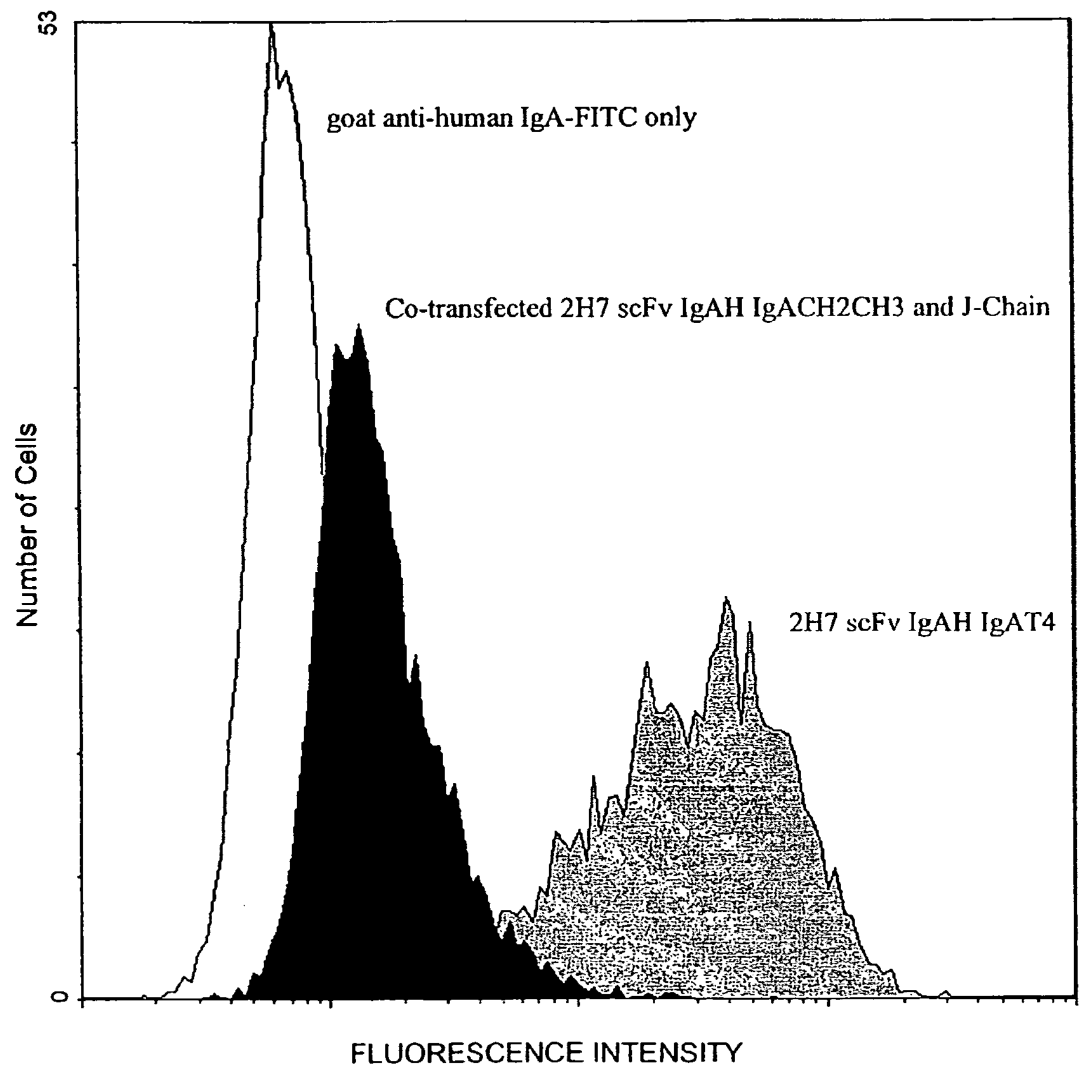
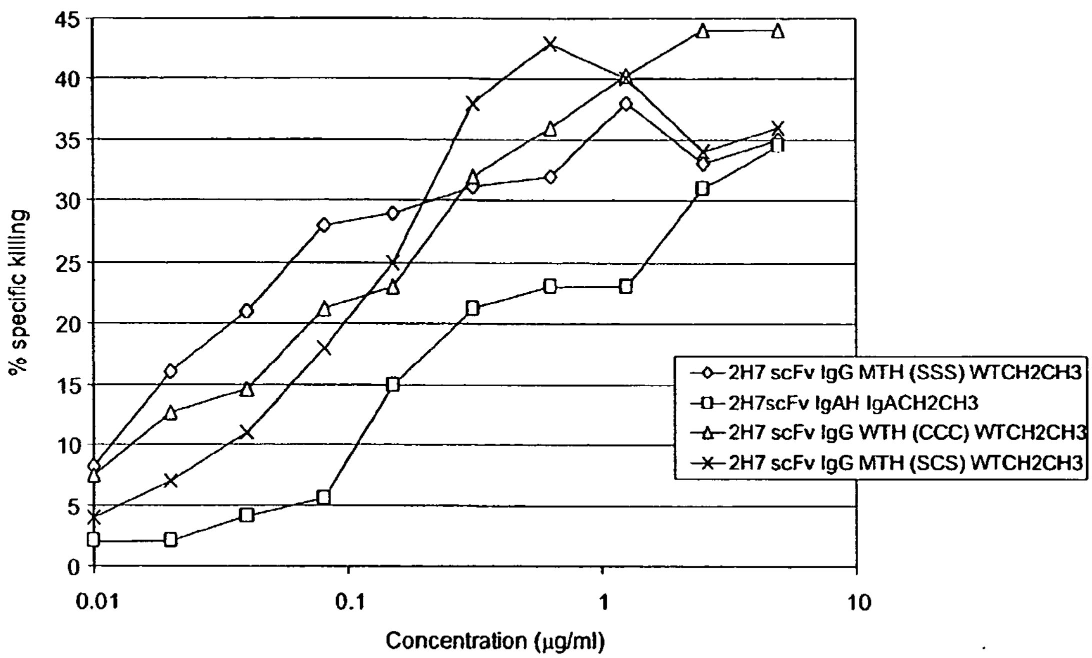
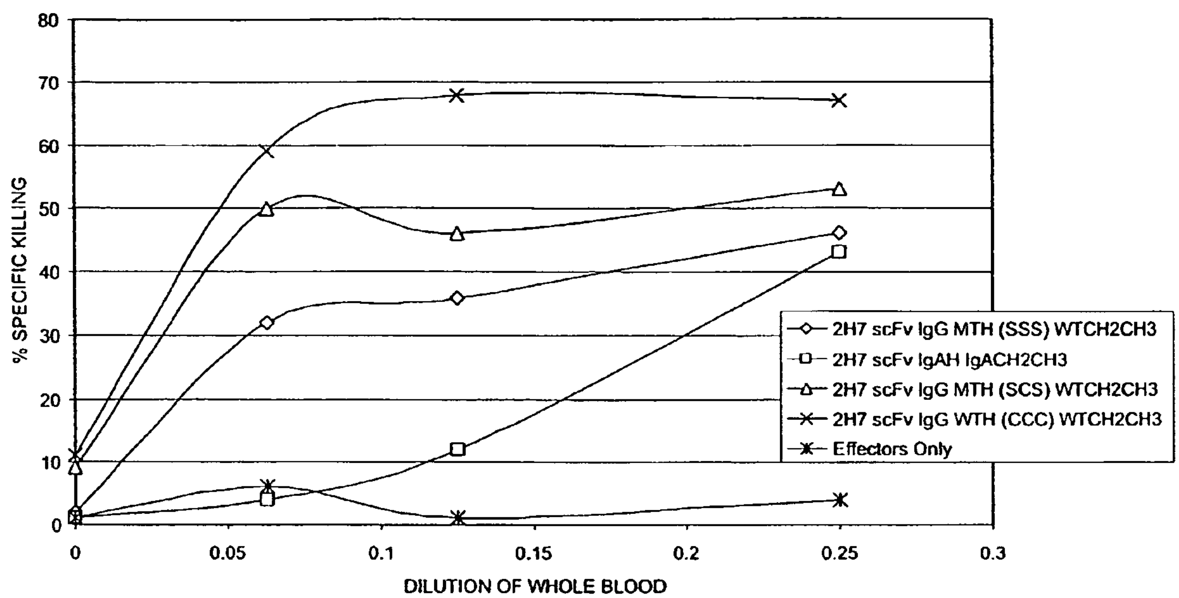

53
0
Number of Cells
goat anti-human IgA-FITC only
Co-transfected 2H7 scFv IgAH IgACH2CH3 and J-Chain
2H7 scFv IgAH IgAT4
FLUORESCENCE INTENSITY

Figure 37



% specific killing
45
40
35
30
25
20
15
10
5
0
0.01
0.1
1
10
Concentration (µg/ml)
2H7 scFv IgG MTH (SSS) WTCH2CH3
2H7scFv IgAH IgACH2CH3
2H7 scFv IgG WTH (CCC) WTCH2CH3
2H7 scFv IgG MTH (SCS) WTCH2CH3

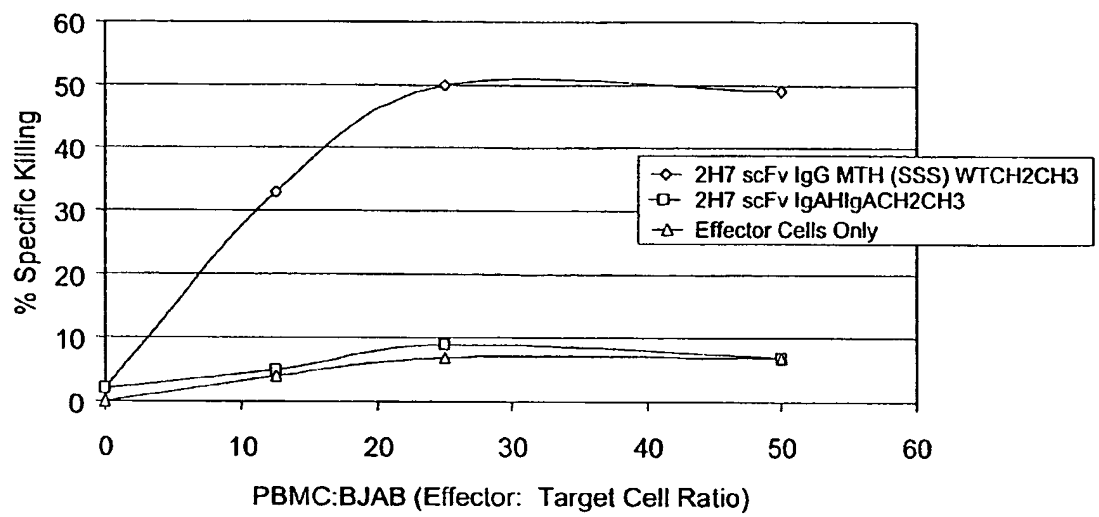
Figure 38



80
70
60
50
40
30
20
10
0
% SPECIFIC KILLING
0
0.05
0.1
0.15
0.2
0.25
0.3
DILUTION OF WHOLE BLOOD
2H7 scFv IgG MTH (SSS) WTCH2CH3
2H7 scFv IgAH IgACH2CH3
2H7 scFv IgG MTH (SCS) WTCH2CH3
2H7 scFv IgG WTH (CCC) WTCH2CH3
Effectors Only

Figures 39A and 39B

A



B

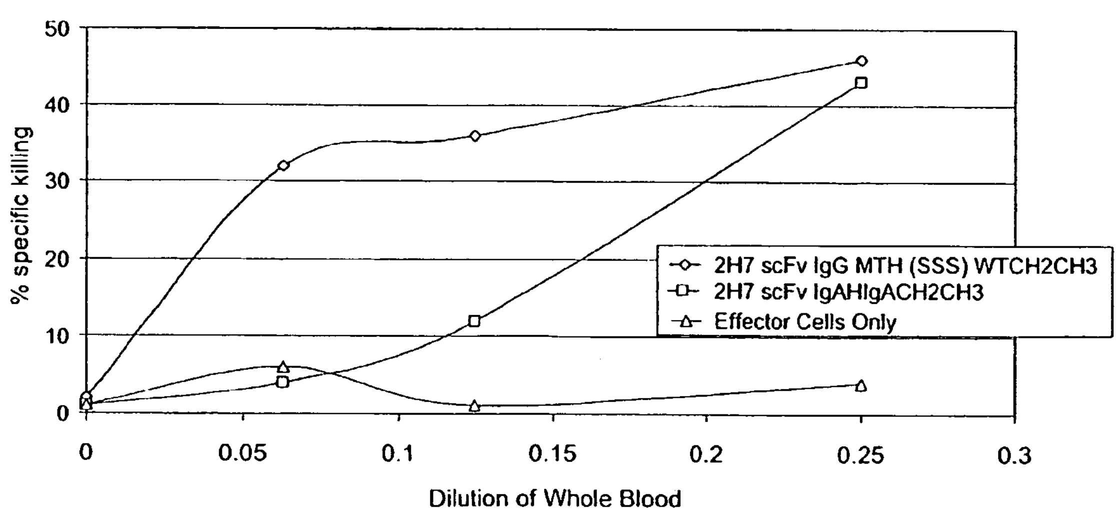
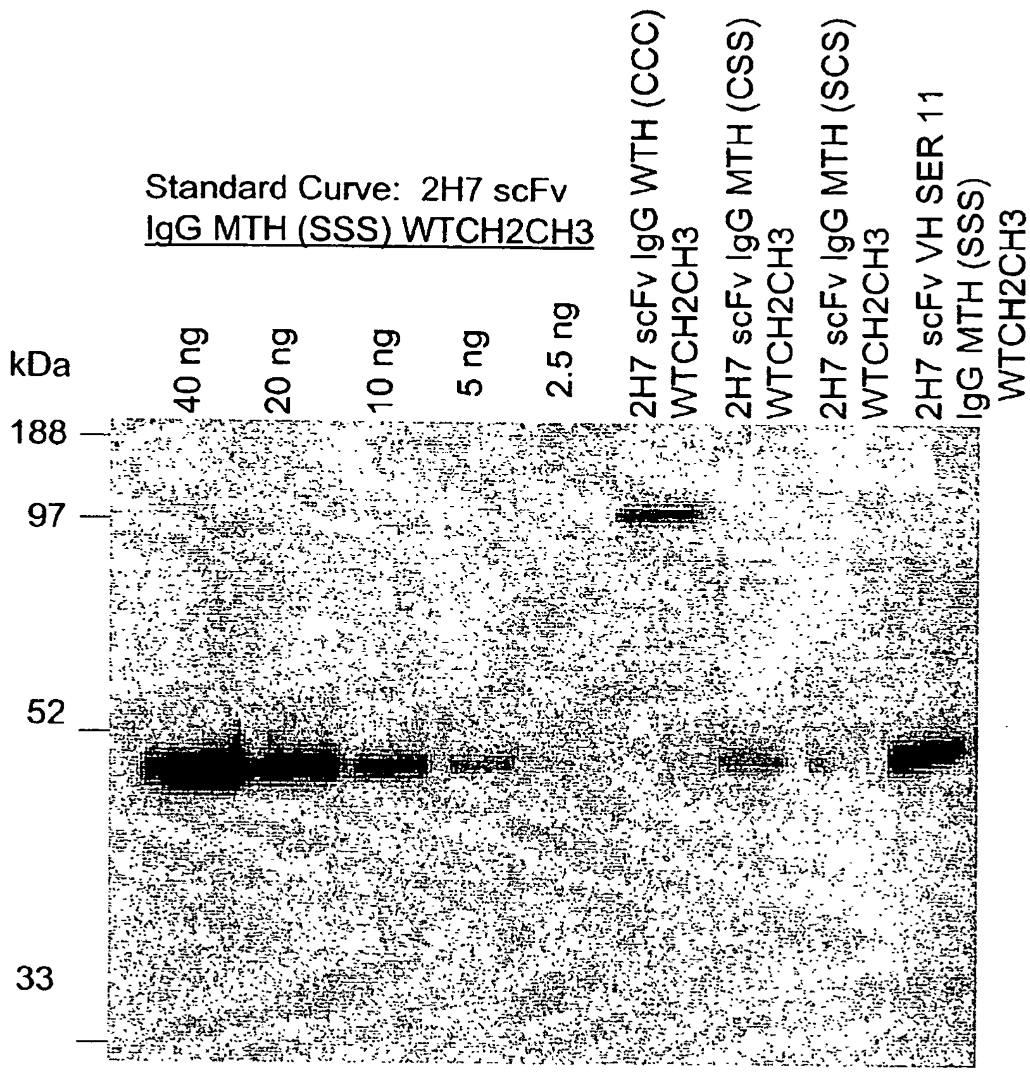


Figure 40


Standard Curve: 2H7 scFv
IgG MTH (SSS) WTCH2CH3
kDa
40 ng
20 ng
10 ng
5 ng
2.5 ng
2H7 scFv IgG WTH (CCC) WTCH2CH3
2H7 scFv IgG MTH (CSS) WTCH2CH3
2H7 scFv IgG MTH (SCS) WTCH2CH3
2H7 scFv VH SER 11 IgG MTH (SSS) WTCH2CH3
188
97
52
33

Figures 41A, 41B and 41C

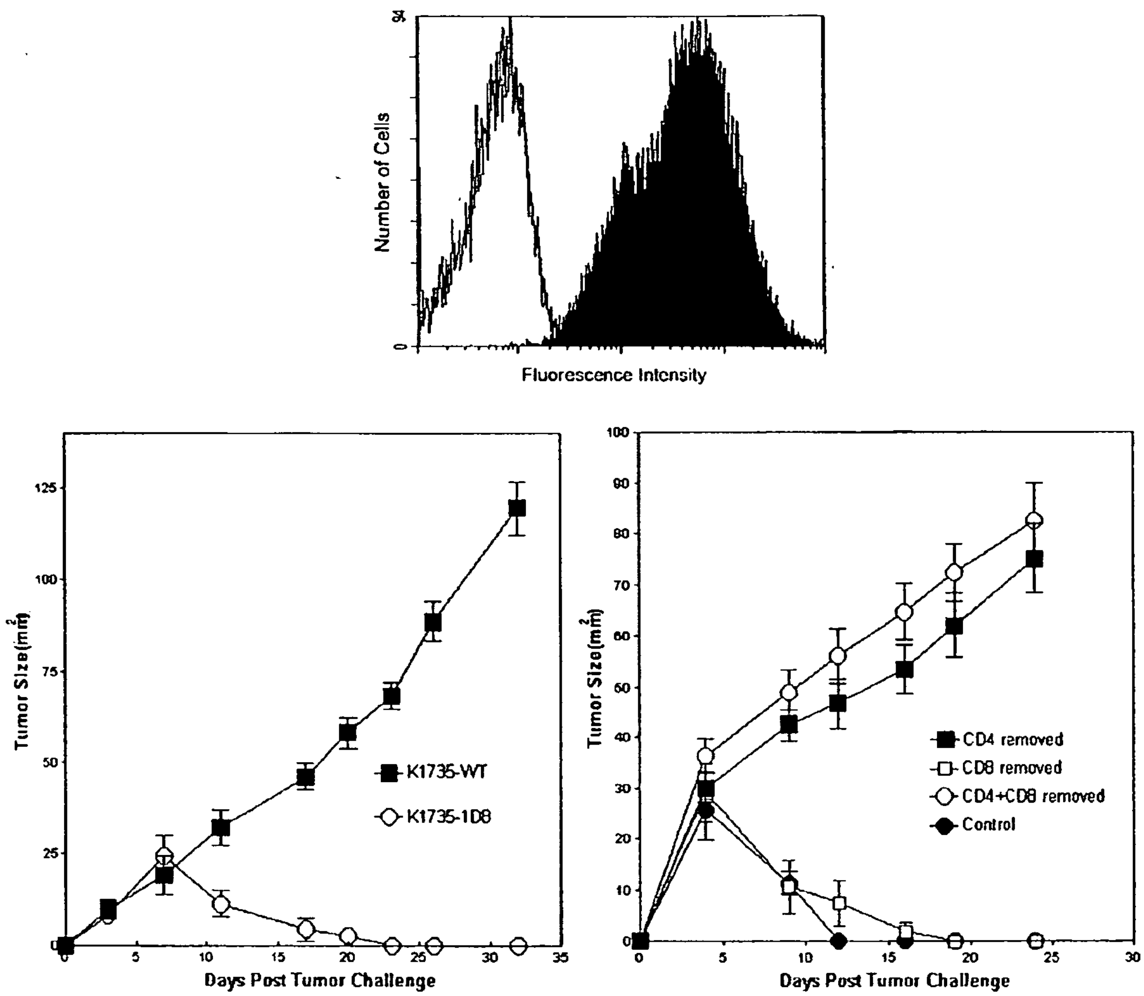


Figure 42

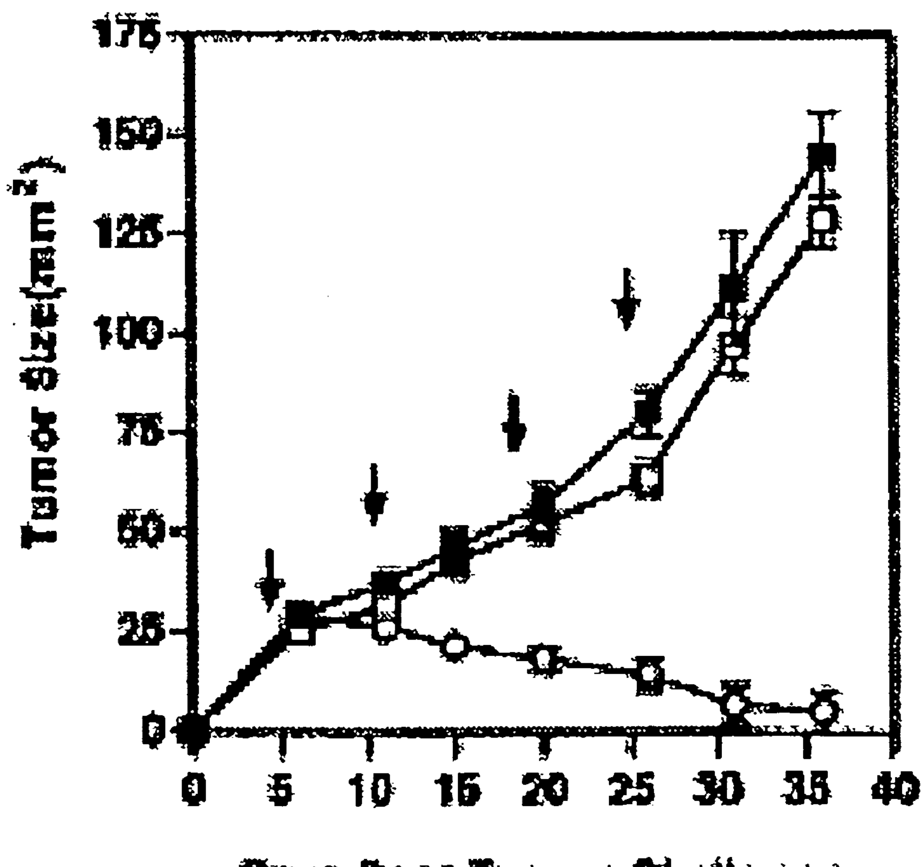

Tumor Size (mm²)
175
150
125
100
75
50
25
0
0
5
10
15
20
25
30
35
40
Days Post Tumor Challenge

Figure 43

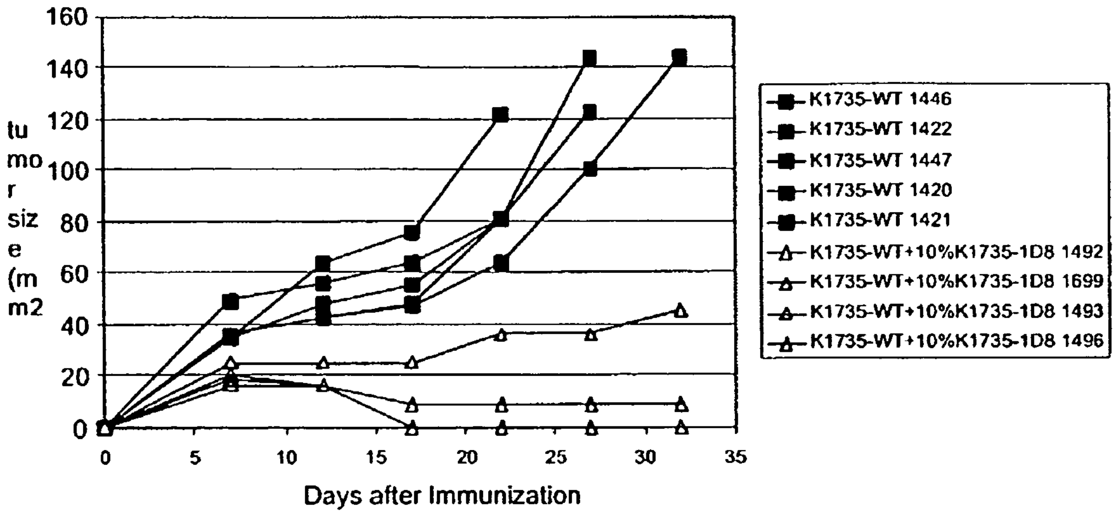

160
140
120
100
80
60
40
20
0
tu
mo
r
siz
e
(m
m2
0
5
10
15
20
25
30
35
Days after Immunization
K1735-WT 1446
K1735-WT 1422
K1735-WT 1447
K1735-WT 1420
K1735-WT 1421
K1735-WT+10%K1735-1D8 1492
K1735-WT+10%K1735-1D8 1699
K1735-WT+10%K1735-1D8 1493
K1735-WT+10%K1735-1D8 1496

Figure 44

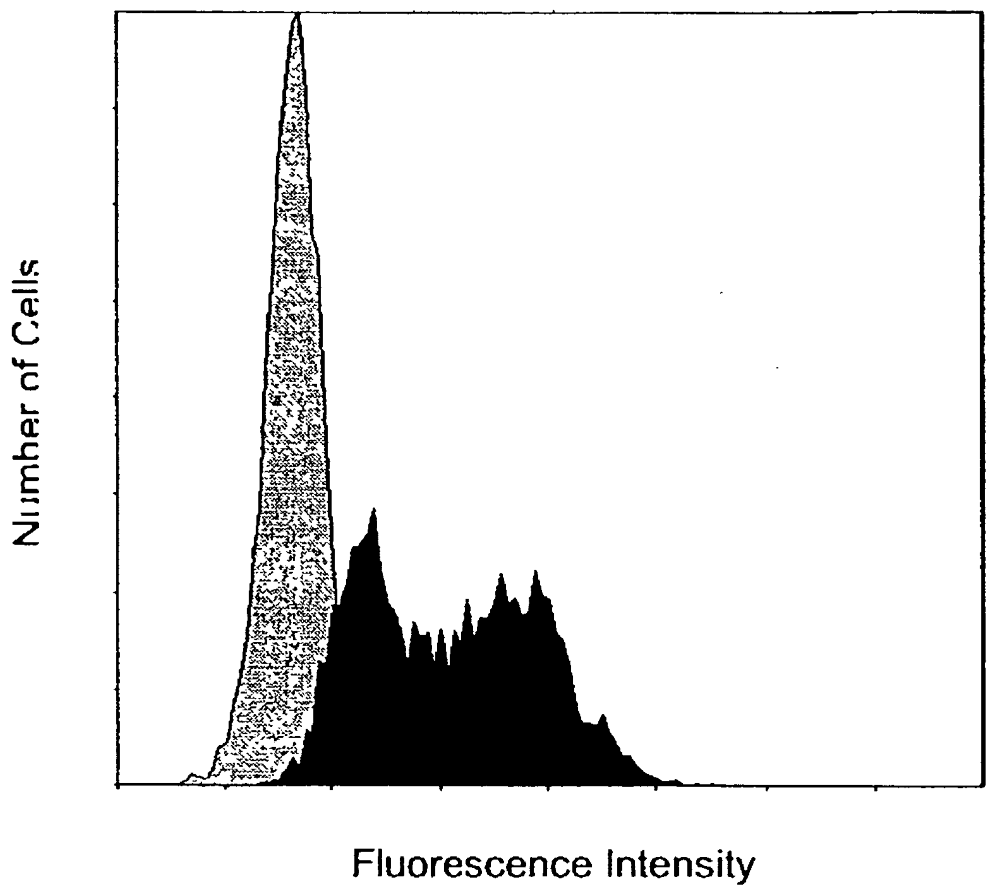


Figure 45

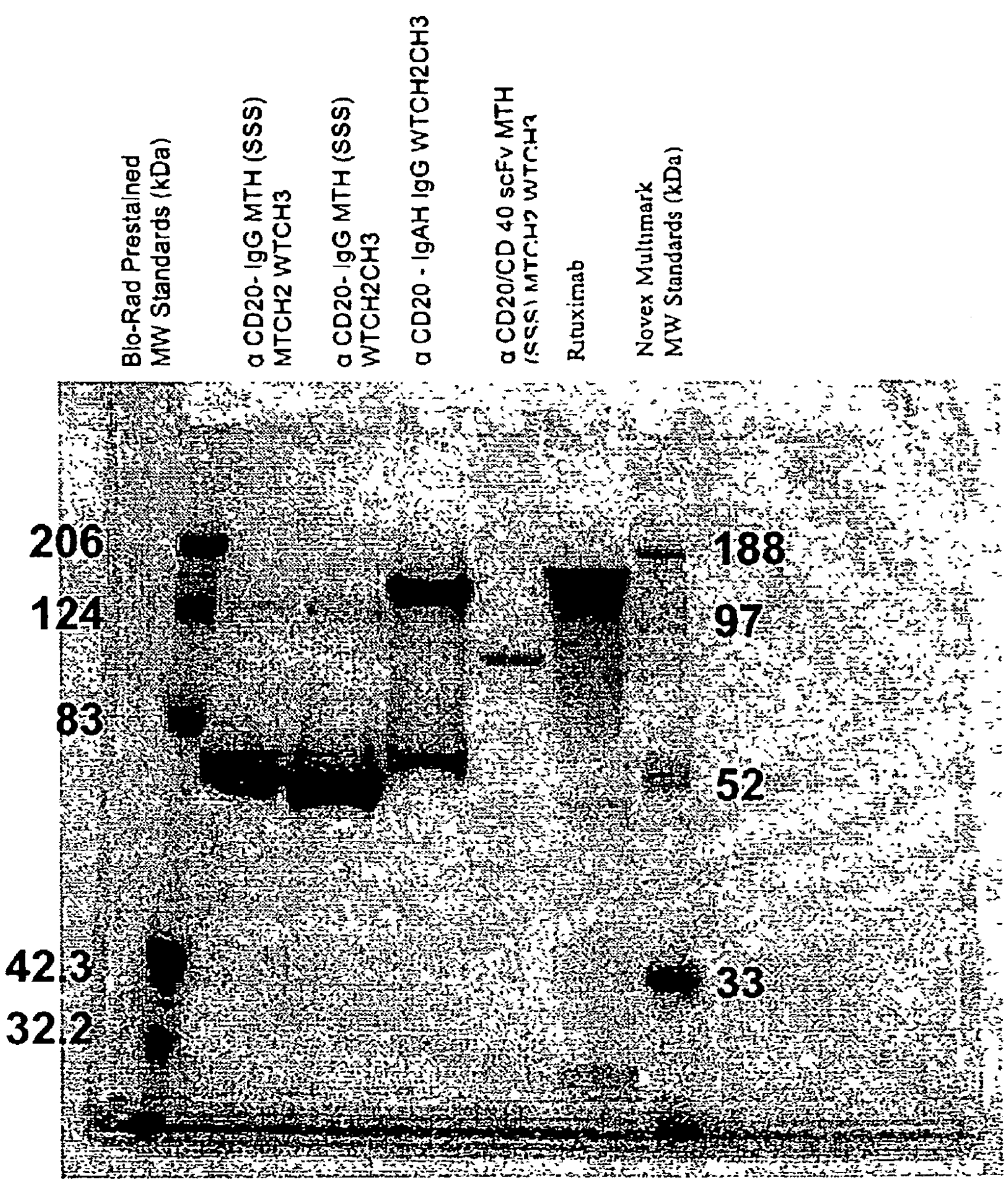
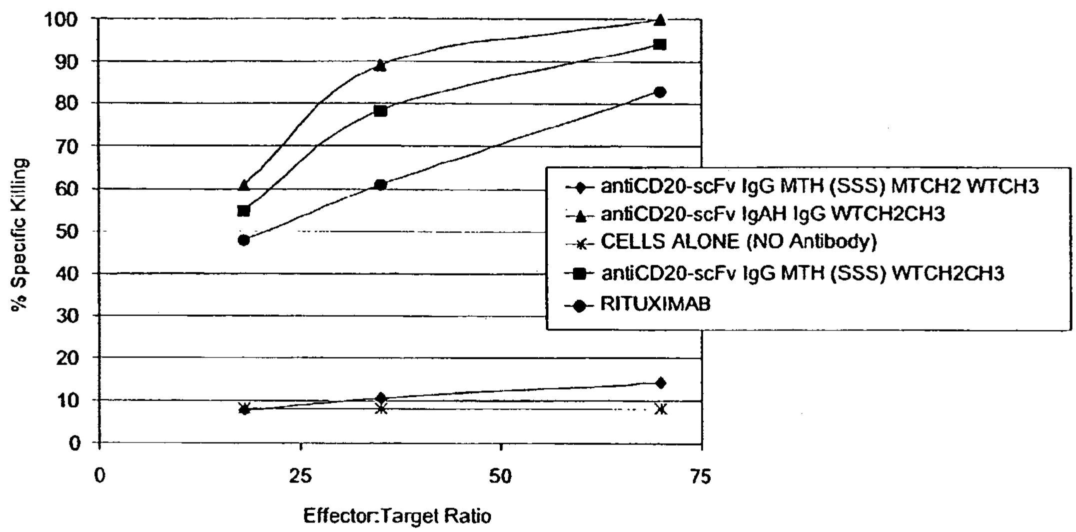
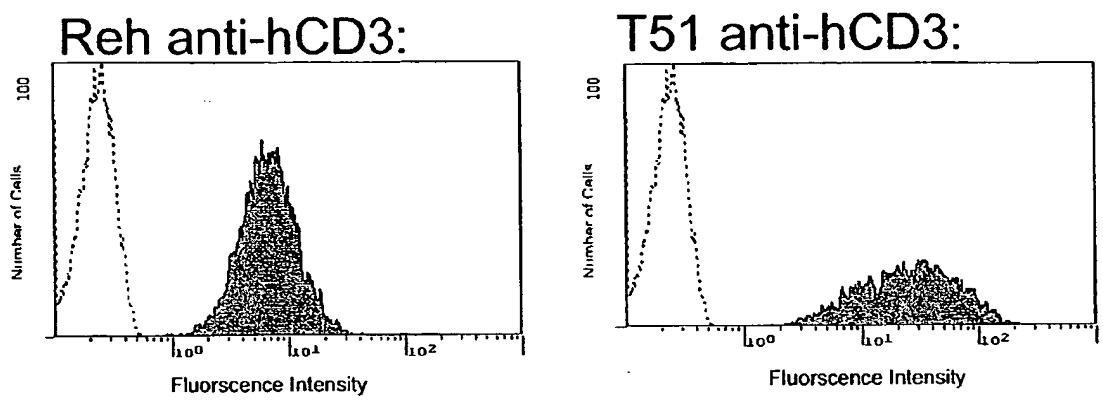

Bio-Rad Prestained MW Standards (kDa)
α CD20- IgG MTH (SSS) MTCH2 WTCH3
α CD20- IgG MTH (SSS) WTCH2CH3
α CD20 - IgAH IgG WTCH2CH3
α CD20/CD 40 scFv MTH (SSS) MTCH2 WTCH3
Rituximab
Novex Multimark MW Standards (kDa)
206
124
83
42.3
32.2
188
97
52
33

Figure 46



100
90
80
70
60
50
40
30
20
10
0
% Specific Killing
0
25
50
75
Effector:Target Ratio
antiCD20-scFv IgG MTH (SSS) MTCH2 WTCH3
antiCD20-scFv IgAH IgG WTCH2CH3
CELLS ALONE (NO Antibody)
antiCD20-scFv IgG MTH (SSS) WTCH2CH3
RITUXIMAB

Figure 47


Reh anti-hCD3:
100
Number of Cells
10⁰
10¹
10²
Fluorscence Intensity
T51 anti-hCD3:
100
Number of Cells
10⁰
10¹
10²
Fluorscence Intensity

Figures 48A and 48B

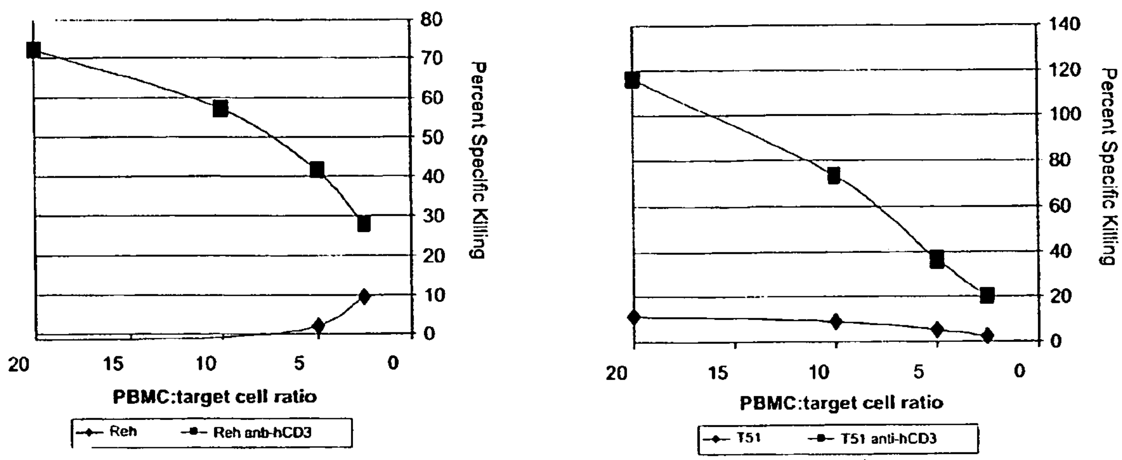
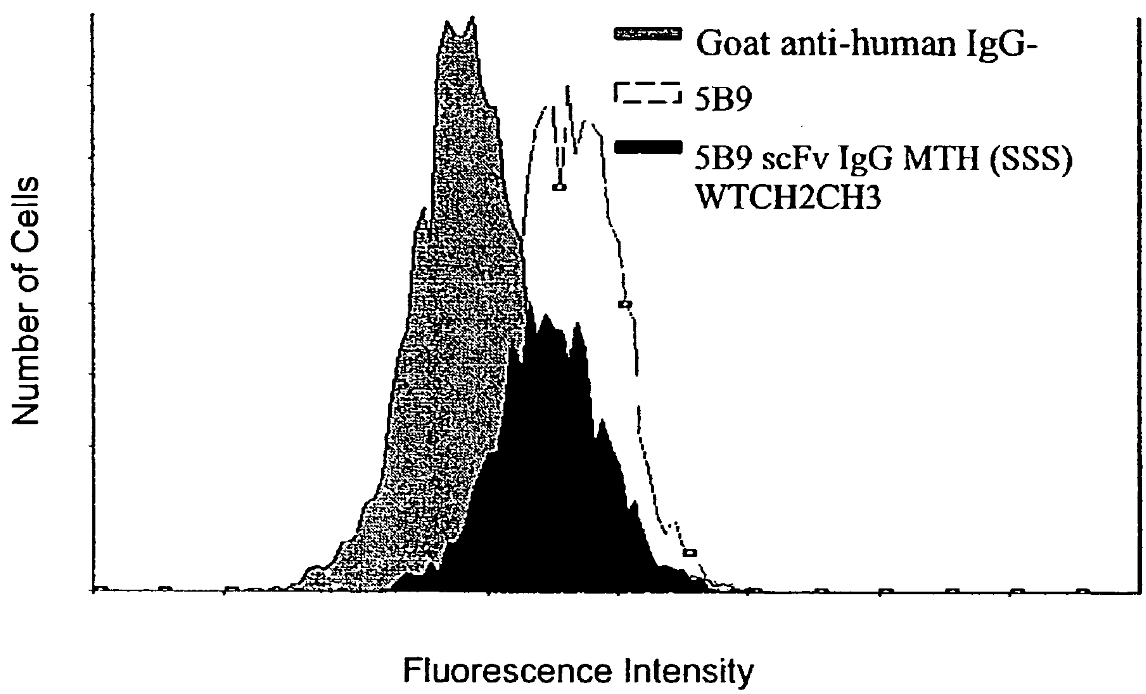


Figure 49


Goat anti-human IgG-
5B9
5B9 scFv IgG MTH (SSS) WTCH2CH3
Number of Cells
Fluorescence Intensity

BINDING DOMAIN-IMMUNOGLOBULIN FUSION PROTEINS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application is a Continuation-in-Part of U.S. Ser. No. 10/053,530, filed Jan. 17, 2002, which is incorporated herein by reference in its entirety and which claims the benefit of priority of U.S. Provisional Application No. 60/367,358 (formerly U.S. Ser. No. 09/765,208, filed Jan. 17, 2001), also incorporated herein by reference in its entirety and for which a Petition to Convert a Non-Provisional Application to a Provisional Application was filed on Jan. 16, 2002, and granted on Jun. 6, 2002. This application also claims the benefit of priority of U.S. Provisional Application No. 60/385,691 filed on Jun. 3, 2002, and entitled "Recombinant Signaling Receptors for Tumor Gene Therapy", which is also incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The present invention relates generally to immunologically active, recombinant binding proteins, and in particular, to molecularly engineered binding domain-immunoglobulin fusion proteins, including single chain Fv-immunoglobulin fusion proteins. The present invention also relates to compositions and methods for treating malignant conditions and B-cell disorders, including diseases characterized by autoantibody production.

[0003] An immunoglobulin molecule is a multimeric protein composed of two identical light chain polypeptides and two identical heavy chain polypeptides (H_2L_2) that are joined into a macromolecular complex by interchain disulfide bonds. Intrachain disulfide bonds join different areas of the same polypeptide chain, which results in the formation of loops that, along with adjacent amino acids, constitute the immunoglobulin domains. At the amino-terminal portion, each light chain and each heavy chain has a single variable region that shows considerable variation in amino acid composition from one antibody to another. The light chain variable region, V_L, associates with the variable region of a heavy chain, V_H, to form the antigen binding site of the immunoglobulin, Fv. Light chains have a single constant region domain and heavy chains have several constant region domains. Classes IgG, IgA, and IgD have three constant region domains, which are designated CH1, CH2, and CH3, and the IgM and IgE classes have four constant region domains, CH1, CH2, CH3 and CH4. Immunoglobulin structure and function are reviewed, for example, in Harlow et al., Eds., *Antibodies: A Laboratory Manual*, Chapter 14, Cold Spring Harbor Laboratory, Cold Spring Harbor (1988).

[0004] The heavy chains of immunoglobulins can be divided into three functional regions: Fd (fragment comprising VH and CH1), hinge, and Fc (fragment crystallizable, derived from constant regions). The Fd region comprises the V_H and CH1 domains and in combination with the light chain forms Fab, the antigen-binding fragment. The Fc fragment is generally considered responsible for the effector functions of an immunoglobulin, such as complement fixation and binding to Fc receptors. The hinge region, found in IgG, IgA, and IgD classes, acts as a flexible spacer, allowing the Fab portion to move freely in space. In contrast to the constant regions, the hinge domains are structurally diverse, varying in both sequence and length among immunoglobulin classes and subclasses. For example, three human IgG subclasses, IgG1, IgG2, and IgG4, have hinge regions of 12-15 amino acids, while IgG3-derived hinge regions can comprise approximately 62 amino acids, including around 21 proline residues and around 11 cysteine residues.

[0005] According to crystallographic studies, the immunoglobulin hinge region can be further subdivided functionally into three regions: the upper hinge, the core, and the lower hinge (Shin et al., *Immunological Reviews* 130:87 (1992)). The upper hinge includes amino acids from the carboxyl end of CH1 to the first residue in the hinge that restricts motion, generally the first cysteine residue that forms an interchain disulfide bond between the two heavy chains. The length of the upper hinge region correlates with the segmental flexibility of the antibody. The core hinge region contains the inter-heavy chain disulfide bridges, and the lower hinge region joins the amino terminal end of the CH2 domain and includes residues in CH2. (Id.) The core hinge region of human IgG1 contains the sequence Cys-Pro-Pro-Cys which, when dimerized by disulfide bond formation, results in a cyclic octapeptide believed to act as a pivot, thus conferring flexibility. The hinge region may also contain one or more glycosylation sites, which include a number of structurally distinct types of sites for carbohydrate attachment. For example, IgA1 contains five glycosylation sites within a 17 amino acid segment of the hinge region, conferring exceptional resistance of the hinge region polypeptide to intestinal proteases, considered an advantageous property for a secretory immunoglobulin.

[0006] Conformational changes permitted by the structure and flexibility of the immunoglobulin hinge region polypeptide sequence may affect the effector functions of the Fc portion of the antibody. Three general categories of effector functions associated with the Fc region include (1) activation of the classical complement cascade, (2) interaction with effector cells, and (3) compartmentalization of immunoglobulins. The different human IgG subclasses vary in the relative efficacies with which they fix complement, or activate and amplify the steps of the complement cascade (e.g., Kirschfink, 2001 *Immunol. Rev.* 180:177; Chakraborti et al., 2000 *Cell Signal* 12:607; Kohl et al., 1999 *Mol. Immunol.* 36:893; Marsh et al., 1999 *Curr. Opin. Nephrol. Hypertens.* 8:557; Speth et al., 1999 *Wien Klin. Wochenschr.* 111:378). Complement-dependent cytotoxicity (CDC) is believed to be a significant mechanism for clearance of specific target cells such as tumor cells. In general, IgG1 and IgG3 most effectively fix complement, IgG2 is less effective, and IgG4 does not activate complement. Complement activation is initiated by binding of C1q, a subunit of the first component C1 in the cascade, to an antigen-antibody complex. Even though the binding site for C1q is located in the CH2 domain of the antibody, the hinge region influences the ability of the antibody to activate the cascade. For example, recombinant immunoglobulins lacking a hinge region are unable to activate complement. (Shin et al., 1992) Without the flexibility conferred by the hinge region, the Fab portion of the antibody bound to the antigen may not be able to adopt the conformation required to permit C1q to bind to CH2. (See id.) Hinge length and segmental flexibility have been correlated with complement activation; however, the correla-

tion is not absolute. Human IgG3 molecules with altered hinge regions that are as rigid as IgG4 can still effectively activate the cascade.

[0007] The absence of a hinge region, or a lack of a functional hinge region, can also affect the ability of certain human IgG immunoglobulins to bind Fc receptors on immune effector cells. Binding of an immunoglobulin to an Fc receptor facilitates antibody-dependent cell-mediated cytotoxicity (ADCC), which is presumed to be an important mechanism for the elimination of tumor cells. The human IgG Fc receptor (FcR) family is divided into three groups, FcγRI (CD64), which is capable of binding IgG with high affinity, and FcγRII (CD32) and FcγRIII (CD16), both of which are low affinity receptors. The molecular interaction between each of the three receptors and an immunoglobulin has not been defined precisely, but experimental evidence indicates that residues in the hinge proximal region of the CH2 domain are important to the specificity of the interaction between the antibody and the Fc receptor. In addition, IgG1 myeloma proteins and recombinant IgG3 chimeric antibodies that lack a hinge region are unable to bind FcγRI, likely because accessibility to CH2 is decreased. (Shin et al., *Intern. Rev. Immunol.* 10:177, 178-79 (1993)).

[0008] Unusual and apparently evolutionarily unrelated exceptions to the H_2L_2 structure of conventional antibodies occur in some isotypes of the immunoglobulins found in camelids (Hamers-Casterman et al., 1993 *Nature* 363:446; Nguyen et al., 1998 *J. Mol. Biol* 275:413) and in nurse sharks (Roux et al., 1998 *Proc. Nat. Acad. Sci. USA* 95:11804). These antibodies form their antigen-binding pocket using the heavy chain variable region alone. In both species, these variable regions often contain an extended third complementarity determining region (CDR3) to help compensate for the lack of a light chain variable region, and there are frequent disulfide bonds between CDR regions to help stabilize the binding site [Muyldermans et al., 1994 *Prot. Engineer.* 7:1129; Roux et al., 1998]. However, the function of the heavy chain-only antibodies is unknown, and the evolutionary pressure leading to their formation has not been identified. Since camelids, including camels, llamas, and alpacas, also express conventional H_2L_2 antibodies, the heavy chain-only antibodies do not appear to be present in these animals simply as an alternative antibody structure.

[0009] Variable regions (V_HH) of the camelid heavy chain-only immunoglobulins contain amino acid substitutions at several positions outside of the CDR regions when compared with conventional (H_2L_2) heavy chain variable regions. These amino acid substitutions are encoded in the germ line [Nguyen et al., 1998 *J. Mol. Biol* 275:413] and are located at residues that normally form the hydrophobic interface between conventional V_H and V_L domains [Muyldermans et al., 1994 *Prot. Engineer.* 7:1129]. Camelid V_HH recombine with IgG2 and IgG3 constant regions that contain hinge, CH2, and CH3 domains but which lack a CH1 domain [Hamers-Casterman et al., 1993 *Nature* 363:446]. Interestingly, V_HH are encoded by a chromosomal locus distinct from the V_H locus [Nguyen, 1998], indicating that camelid B cells have evolved complex mechanisms of antigen recognition and differentiation. Thus, for example, llama IgG1 is a conventional (H_2L_2) antibody isotype in which V_H recombines with a constant region that contains hinge, CH1, CH2 and CH3 domains, whereas the llama IgG2 and IgG3 are heavy chain-only isotypes that lack CH1 domains and that contain no light chains.

[0010] Monoclonal antibody technology and genetic engineering methods have led to rapid development of immunoglobulin molecules for diagnosis and treatment of human diseases. Protein engineering has been applied to improve the affinity of an antibody for its cognate antigen, to diminish problems related to immunogenicity of administered recombinant polypeptides, and to alter antibody effector functions. The domain structure of immunoglobulins is amenable to recombinant engineering, in that the antigen binding domains and the domains conferring effector functions may be exchanged between immunoglobulin classes (e.g., IgG, IgA, IgE) and subclasses (e.g., IgG1, IgG2, IgG3, etc.).

[0011] In addition, smaller immunoglobulin molecules have been constructed to overcome problems associated with whole immunoglobulin therapy. For instance, single chain immunoglobulin variable region fragment polypeptides (scFv) comprise an immunoglobulin heavy chain variable domain joined via a short linker peptide to an immunoglobulin light chain variable domain (Huston et al. *Proc. Natl. Acad. Sci. USA,* 85: 5879-83, 1988). Because of the small size of scFv molecules, they exhibit very rapid clearance from plasma and tissues and are capable of more effective penetration into tissues than whole immunoglobulins. (see, e.g., Jain, 1990 *Cancer Res.* 50:814s-819s.) An anti-tumor scFv showed more rapid tumor penetration and more even distribution through the tumor mass than the corresponding chimeric antibody (Yokota et al., *Cancer Res.* 52, 3402-08 (1992)). Fusion of an scFv to another molecule, such as a toxin, takes advantage of the specific antigen-binding activity and the small size of an scFv to deliver the toxin to a target tissue. (Chaudary et al., *Nature* 339:394 (1989); Batra et al., *Mol. Cell. Biol.* 11:2200 (1991)).

[0012] Despite the advantages that scFv molecules bring to serotherapy, several drawbacks to this therapeutic approach exist. While rapid clearance of scFv may reduce toxic effects in normal cells, such rapid clearance may prevent delivery of a minimum effective dose to the target tissue. Manufacturing adequate amounts of scFv for administration to patients has been challenging due to difficulties in expression and isolation of scFv that adversely affect the yield. During expression, scFv molecules lack stability and often aggregate due to pairing of variable regions from different molecules. Furthermore, production levels of scFv molecules in mammalian expression systems are low, limiting the potential for efficient manufacturing of scFv molecules for therapy (Davis et al, *J. Biol. Chem.* 265:10410-18 (1990); Traunecker et al., *EMBO J.* 10: 3655-59 (1991)). Strategies for improving production have been explored, including addition of glycosylation sites to the variable regions (e.g., U.S. Pat. No. 5,888,773; Jost et al, *J. Biol. Chem.* 269: 26267-73 (1994)).

[0013] An additional disadvantage to using scFv for therapy is the lack of effector function. An scFv that lacks the cytolytic functions, ADCC and complement dependent-cytotoxicity (CDC), which are typically associated with immunoglobulin constant regions, may be ineffective for treating disease. Even though development of scFv technology began over 12 years ago, currently no scFv products are approved for therapy. Conjugation or fusion of toxins to

scFV has thus been an alternative strategy to provide a potent, antigen-specific molecule, but dosing with such conjugates or chimeras is often limited by excessive and/or non-specific toxicity having its origin in the toxin moiety of such preparations. Toxic effects may include supraphysiological elevation of liver enzymes and vascular leak syndrome, and other undesired effects. In addition, immunotoxins are themselves highly immunogenic after being administered to a host, and host antibodies generated against the immunotoxin limit its potential usefulness in repeated therapeutic treatments of an individual.

[0014] The benefits of immunoglobulin constant region-associated effector functions in the treatment of disease has prompted development of fusion proteins in which immunoglobulin constant region polypeptide sequences are present and nonimmunoglobulin sequences are substituted for the antibody variable region. For example, CD4, the T cell surface protein recognized by HIV, was recombinantly fused to an immunoglobulin Fc effector domain. (See Sensel et al., *Chem. Immunol* 65:129-158 (1997)). The biological activity of such a molecule will depend in part on the class or subclass of the constant region chosen. An IL-2-IgG1 fusion protein effected complement-mediated lysis of IL-2 receptor-bearing cells. (See id.). Use of immunoglobulin constant regions to construct these and other fusion proteins may also confer improved pharmacokinetic properties.

[0015] Diseases and disorders thought to be amenable to some type of immunoglobulin therapy include cancer and immune system disorders. Cancer includes a broad range of diseases, affecting approximately one in four individuals worldwide. Rapid and unregulated proliferation of malignant cells is a hallmark of many types of cancer, including hematological malignancies. Patients with a hematologic malignant condition have benefited most from advances in cancer therapy in the past two decades (Multani et al., *J. Clin. Oncology* 16: 3691-3710, 1998). Although remission rates have increased, most patients still relapse and succumb to their disease. Barriers to cure with cytotoxic drugs include tumor cell resistance and the high toxicity of chemotherapy, which prevents optimal dosing in many patients. New treatments based on targeting with molecules that specifically bind to a malignant cell, including monoclonal antibodies (mAbs), can improve effectiveness without increasing toxicity.

[0016] Since monoclonal antibodies (mAb) were first described in 1975 (Kohler et al., *Nature* 256:495-97 (1975)), many patients have been treated with mAbs that specifically bind to tumor antigens, or antigens expressed on tumor cells. These studies have yielded important lessons regarding the selection of tumor cell surface antigens that are tumor antigens suitable for use as immunotherapy targets. First, it is highly preferable that such a target antigen is not expressed by normal tissues the preservation of which is important to host survival. Fortunately, in the case of hematologic malignancy, malignant cells express many antigens that are not expressed on the surfaces of stem cells or other essential cells. Treatment of a hematologic malignant condition using a therapeutic regimen that depletes both normal and malignant cells of hematological origin has been acceptable where regeneration of normal cells from progenitors can occur after therapy has ended. Second, the target antigen should be expressed on all or virtually all clonogenic populations of tumor cells, and expression should persist despite the selective pressure from immunoglobulin therapy. Thus, a strategy that employs selection of a cell surface idiotype (e.g., a particular idiotope) as a target for therapy of B cell malignancy has been limited by the outgrowth of tumor cell variants with altered surface idiotype expression, even where the antigen exhibits a high degree of tumor selectivity (Meeker et al., *N. Engl. J. Med.* 312:1658-65 (1985)). Third, the selected antigen must traffic properly after an immunoglobulin binds to it. Shedding or internalization of a cell surface target antigen after an immunoglobulin binds to the antigen may allow tumor cells to escape destruction, thus limiting the effectiveness of serotherapy. Fourth, binding of an immunoglobulin to cell surface target antigens that transmit or transduce cellular activation signals may result in improved functional responses to immunotherapy in tumor cells, and can lead to growth arrest and/or apoptosis. While all of these properties are important, the triggering of apoptosis after an immunoglobulin binds to the target antigen may be a critical factor in achieving successful serotherapy.

[0017] Antigens that have been tested as targets for serotherapy of B and T cell malignancies include Ig idiotype (Brown et al., *Blood* 73:651-61 (1989)), CD19 (Hekman et al., *Cancer Immunol. Immunother.* 32:364-72 (1991); Vlasveld et al., *Cancer Immunol. Immunother.* 40: 37-47 (1995)), CD20 (Press et al., *Blood* 69: 584-91 (1987); Maloney et al., *J. Clin. Oncol.* 15:3266-74, (1997)) CD21 (Scheinberg et. al., *J. Clin. Oncol.* 8:792-803, (1990)), CD5 (Dillman et. al., *J. Biol. Respn. Mod.* 5:394-410 (1986)), and CD52 (CAMPATH) (Pawson et al., *J. Clin. Oncol.* 15:2667-72, (1997)). Of these, the most success has been obtained using CD20 as a target for therapy of B cell lymphomas. Each of the other targets has been limited by the biological properties of the antigen. For example, surface idiotype can be altered through somatic mutation, allowing tumor cell escape. As other examples, CD5, CD21, and CD19 are rapidly internalized after mAb binding, allowing tumor cells to escape destruction unless mAbs are conjugated with toxin molecules. CD22 is expressed on only a subset of B cell lymphomas, thereby limiting its usefulness, while CD52 is expressed on both T cells and B cells and may therefore generate counterproductive immunosuppression by effecting selective T cell depletion.

[0018] CD20 fulfills the basic criteria described above for selection of an appropriate target antigen for therapy of a B cell malignant condition. Treatment of patients with low grade or follicular B cell lymphoma using chimeric CD20 mAb induces partial or complete responses in many patients (McLaughlin et al, *Blood* 88:90a (abstract, suppl. 1) (1996); Maloney et al, *Blood* 90: 2188-95 (1997)). However, tumor relapse commonly occurs within six months to one year. Therefore, further improvements in serotherapy are needed to induce more durable responses in low grade B cell lymphoma, and to allow effective treatment of high grade lymphoma and other B cell diseases.

[0019] One approach to improving CD20 serotherapy has been to target radioisotopes to B cell lymphomas using mAbs specific for CD20. While the effectiveness of therapy is increased, associated toxicity from the long in vivo half-life of the radioactive antibody increases also, sometimes requiring that the patient undergo stem cell rescue (Press et al., *N. Eng. J. Med.* 329: 1219-1224, 1993; Kaminski et al., *N. Eng. J. Med.* 329:459-65 (1993)). MAbs to

CD20 have been cleaved with proteases to yield $F(ab')_2$ or Fab fragments prior to attachment of the radioisotope. This improves penetration of the radioisotope conjugate into the tumor, and shortens the in vivo half-life, thus reducing the toxicity to normal tissues. However, the advantages of effector functions, including complement fixation and/or ADCC that would otherwise be provided by the Fc region of the CD20 mAb, are lost since the Fab preparations lack immunoglogulin Fc domains. Therefore, for improved delivery of radioisotopes, a strategy is needed to make a CD20 mAb derivative that retains Fc-dependent effector functions but which is smaller in size, thereby increasing tumor penetration and shortening mAb half-life.

[0020] CD20 was the first human B cell lineage-specific surface molecule identified by a monoclonal antibody, but the function of CD20 in B cell biology is still incompletely understood. CD20 is a non-glycosylated, hydrophobic 35 kDa B cell transmembrane phosphoprotein that has both amino and carboxy ends situated in the cytoplasm (Einfeld et al, *EMBO J.* 7:711-17 (1988)). Natural ligands for CD20 have not been identified. CD20 is expressed by all normal mature B cells, but is not expressed by precursor B cells.

[0021] CD20 mAbs deliver signals to normal B cells that affect viability and growth (Clark et al., *Proc. Natl. Acad. Sci. USA* 83:4494-98 (1986)), and extensive cross-linking of CD20 can induce apoptosis in B lymphoma cell lines (Shan et al., *Blood* 91:1644-52 (1998)). Cross-linking of CD20 on the cell surface increases the magnitude and enhances the kinetics of signal transduction, for example, as detected by measuring tyrosine phosphorylation of cellular substrates (Deans et al., *J. Immunol.* 146:846-53 (1993)). Significantly, apoptosis in Ramos B lymphoma cells can also be induced by FcR cross-linking CD20 mAbs bound to the Ramos cell surfaces, by the addition of Fc-receptor positive cells (Shan et al., *Blood* 91: 1644-52 (1998)). Therefore, in addition to cellular depletion by complement and ADCC mechanisms, Fc-receptor binding by CD20 mAbs in vivo can promote apoptosis of malignant B cells by CD20 cross-linking. This theory is consistent with experiments showing that effectiveness of CD20 therapy of human lymphoma in a SCID mouse model was dependent upon Fc-receptor binding by the CD20 mAb (Funakoshi et al., *J. Immunotherapy* 19:93-101 (1996)).

[0022] The CD20 polypeptide contains four transmembrane domains (Einfeld et al., *EMBO J.* 7: 711-17, (1988); Stamenkovic et al., *J. Exp. Med.* 167:1975-80 (1988); Tedder et. al., *J. Immunol.* 141:4388-4394 (1988)). The multiple membrane spanning domains prevent CD20 internalization after antibody binding. This property of CD20 was recognized as an important feature for effective therapy of B cell malignancies when a murine CD20 mAb, 1F5, was injected into patients with B cell lymphoma, resulting in significant depletion of malignant cells and partial clinical responses (Press et al., *Blood* 69: 584-91 (1987)).

[0023] Because normal mature B cells also express CD20, normal B cells are depleted during CD20 antibody therapy (Reff, M. E. et al, *Blood* 83: 435-445, 1994). However, after treatment is completed, normal B cells are regenerated from CD20 negative B cell precursors; therefore, patients treated with anti-CD20 therapy do not experience significant immunosuppression. Depletion of normal B cells may also be beneficial in diseases that involve inappropriate production of autoantibodies or other diseases where B cells may play a role. A chimeric mAb specific for CD20, consisting of heavy and light chain variable regions of mouse origin fused to human IgG1 heavy chain and human kappa light chain constant regions, retained binding to CD20 and the ability to mediate ADCC and to fix complement (Liu et al., *J. Immunol.* 139:3521-26 (1987); Robinson et al., U.S. Pat. No. 5,500,362). This work led to development of a chimeric CD20 mAb, Rituximab™, currently approved by the U.S. Food and Drug Administration for approval for therapy of B cell lymphomas. While clinical responses are frequently observed after treatment with Rituximab™, patients often relapse after about 6-12 months.

[0024] High doses of Rituximab™ are required for intravenous injection because the molecule is large, approximately 150 kDa, and diffusion is limited into the lymphoid tissues where many tumor cells reside. The mechanism of anti-tumor activity of Rituximab™ is thought to be a combination of several activities, including ADCC, complement fixation, and triggering of signals that promote apoptosis in malignant B cells. The large size of Rituximab™ prevents optimal diffusion of the molecule into lymphoid tissues that contain malignant B cells, thereby limiting these anti-tumor activities. As discussed above, cleavage of CD20 mAbs with proteases into Fab or $F(ab')_2$ fragments makes them smaller and allows better penetration into lymphoid tissues, but the effector functions important for anti-tumor activity are lost. While CD20 mAb fragments may be more effective than intact antibody for delivery of radioisotopes, it would be desirable to construct a CD20 mAb derivative that retains the effector functions of the Fc portion, but that has a smaller molecular size, facilitating better tumor penetration and resulting in a shorter half-life.

[0025] CD20 is expressed by many malignant cells of B cell origin, including B cell lymphoma and chronic lymphocytic leukemia (CLL). CD20 is not expressed by malignancies of pre-B cells, such as acute lymphoblastic leukemia. CD20 is therefore a good target for therapy of B cell lymphoma, CLL, and other diseases in which B cells are involved in the pathogenesis and/or progression of disease. Other B cell disorders include autoimmune diseases in which autoantibodies are produced during or after the differentiation of B cells into plasma cells. Examples of B cell disorders include autoimmune thyroid disease, including Graves' disease and Hashimoto's thyroiditis, rheumatoid arthritis, systemic lupus erythematosus (SLE), Sjogrens syndrome, immune thrombocytopenic purpura (ITP), multiple sclerosis (MS), myasthenia gravis (MG), psoriasis, scleroderma, and inflammatory bowel disease, including Crohn's disease and ulcerative colitis.

[0026] In view of the foregoing, there is clearly a need for improved compositions and methods to treat malignant conditions in general, and in particular B cell disorders. As described in greater detail herein, the compositions and methods of the present invention overcome the limitations of the prior art by providing a binding domain-immunoglobulin fusion protein that specifically binds to an antigen and that is capable of mediating ADCC or complement fixation. Furthermore, the compositions and methods offer other related advantages.

SUMMARY OF THE INVENTION

[0027] It is an aspect of the present invention to provide a binding domain-immunoglobulin fusion protein, comprising

(a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein said hinge region polypeptide is selected from the group consisting of (i) a wild-type human IgG1 immunoglobulin hinge region polypeptide, (ii) a mutated human IgG1 immunoglobulin hinge region polypeptide that is derived from a wild-type immunoglobulin hinge region polypeptide having three or more cysteine residues, wherein said mutated human IgG1 immunoglobulin hinge region polypeptide contains two cysteine residues and wherein a first cysteine of the wild-type hinge region is not mutated, (iii) a mutated human IgG1 immunoglobulin hinge region polypeptide that is derived from a wild-type immunoglobulin hinge region polypeptide having three or more cysteine residues, wherein said mutated human IgG1 immunoglobulin hinge region polypeptide contains no more than one cysteine residue, and (iv) a mutated human IgG1 immunoglobulin hinge region polypeptide that is derived from a wild-type immunoglobulin hinge region polypeptide having three or more cysteine residues, wherein said mutated human IgG1 immunoglobulin hinge region polypeptide contains no cysteine residues; (b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide; and (c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein (1) the binding domain-immunoglobulin fusion protein is capable of at least one immunological activity selected from the group consisting of antibody dependent cell-mediated cytotoxicity and complement fixation, and (2) the binding domain polypeptide is capable of specifically binding to an antigen.

[0028] In certain embodiments the immunoglobulin hinge region polypeptide is a mutated hinge region polypeptide and exhibits a reduced ability to dimerize, relative to a wild-type human immunoglobulin G hinge region polypeptide. In certain embodiments the binding domain polypeptide comprises at least one immunoglobulin variable region polypeptide that is selected an immunoglobulin light chain variable region polypeptide or an immunoglobulin heavy chain variable region polypeptide. In certain further embodiments the binding domain-immunoglobulin fusion protein comprises an immunoglobulin heavy chain variable region polypeptide, wherein the heavy chain variable region polypeptide is a human immunoglobulin heavy chain variable region polypeptide comprising a mutation at an amino acid at a location corresponding to amino acid position 11 in SEQ ID NO:______. In certain other further embodiments the fusion protein comprises a polypeptide having a sequence selected from SEQ ID NOS:______ or SEQ ID NO:______. In certain embodiments the immunoglobulin variable region polypeptide is derived from a human immunoglobulin, and in certain other embodiments the immunoglobulin variable region polypeptide comprises a humanized immunoglobulin polypeptide sequence. In certain embodiments the immunoglobulin variable region polypeptide is derived from a murine immunoglobulin.

[0029] According to certain embodiments of the present inventin, the binding domain polypeptide comprises (a) at least one immunoglobulin light chain variable region polypeptide; (b) at least one immunoglobulin heavy chain variable region polypeptide; and (c) at least one linker polypeptide that is fused to the polypeptide of (a) and to the polypeptide of (b). In certain further embodiments the immunoglobulin light chain variable region and heavy chain variable region polypeptides are derived from human immunoglobulins, and in certain other further embodiments the linker polypeptide comprises at least one polypeptide having as an amino acid sequence Gly-Gly-Gly-Gly-Ser [SEQ ID NO:______]. In other embodiments the linker polypeptide comprises at least three repeats of a polypeptide having as an amino acid sequence Gly-Gly-Gly-Gly-Ser [SEQ ID NO:______]. In other embodiments the linker comprises a glycosylation site, which in certain further embodiments is an asparagine-linked glycosylation site, an O-linked glycosylation site, a C-mannosylation site, a glypiation site or a phosphoglycation site. In another embodiment at least one of the immunoglobulin heavy chain CH2 constant region polypeptide and the immunoglobulin heavy chain CH3 constant region polypeptide is derived from a human immunoglobulin heavy chain. In another embodiment the immunoglobulin heavy chain constant region CH2 and CH3 polypeptides are of an isotype that is human IgG or human IgA. In certain other embodiments the antigen is CD19, CD20, CD22, CD37, CD40, L6, CD2, CD28, CD30, CD40, CD50 (ICAM3), CD54 (ICAM1), CD80, CD86, B7-H1, CD134 (OX40), CD137 (41BB), CD152 (CTLA-4), CD153 (CD30 ligand), CD154 (CD40 ligand), ICOS, CD19, CD3, CD4, CD25, CD8, CD11b, CD14, CD25, CD56 or CD69. In another embodiment the binding domain polypeptide comprises a CD154 extracellular domain. In still another embodiment the binding domain polypeptide comprises a CD154 extracellular domain and at least one immunoglobulin variable region polypeptide. In another embodiment the binding domain polypeptide comprises a CTLA-4 extracellular domain, and in further embodiments at least one of the immunoglobulin heavy chain constant region polypeptides selected from a CH2 constant region polypeptide and a CH3 constant region polypeptide is a human IgG1 constant region polypeptide. In another further embodiment at least one of the immunoglobulin heavy chain constant region polypeptides selected from a CH2 constant region polypeptide and a CH3 constant region polypeptide is a human IgA constant region polypeptide.

[0030] Turning to another embodiment, the present invention provides a binding domain-immunoglobulin fusion protein, comprising (a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide; (b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide; and (c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein (1) the binding domain polypeptide comprises a CTLA-4 extracellular domain that is capable of specifically binding to at least one CTLA-4 ligand selected from the group consisting of CD80 and CD86, (2) the immunoglobulin hinge region polypeptide comprises a polypeptide that is selected from the group consisting of a human IgA hinge region polypeptide and a human IgG1 hinge region polypeptide, (3) the immunoglobulin heavy chain CH2 constant region polypeptide comprises a polypeptide that is selected from the group consisting of a human IgA heavy chain CH2 constant region polypeptide and a human IgG1 heavy chain CH2 constant region polypeptide, (4) the immunoglobulin heavy chain CH3 constant region polypeptide comprises a polypeptide that is selected from the group consisting of a human IgA heavy chain CH3 constant region polypeptide and a human IgG1 heavy chain CH3 constant region polypeptide, and (5) the

binding domain-immunoglobulin fusion protein is capable of at least one immunological activity selected from the group consisting of antibody dependent cell-mediated cytotoxicity and complement fixation.

[0031] In another embodiment the present invention provides a binding domain-immunoglobulin fusion protein, comprising (a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein said hinge region polypeptide comprises a human IgE hinge region polypeptide; (b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide, wherein said CH2 constant region polypeptide comprises a human IgE CH2 constant region polypeptide; and (c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein said CH3 constant region polypeptide comprises a human IgE CH3 constant region polypeptide wherein (1) the binding domain-immunoglobulin fusion protein is capable of at least one immunological activity selected from antibody dependent cell-mediated cytotoxicity and induction of an allergic response mechanism, and (2) the binding domain polypeptide is capable of specifically binding to an antigen. In a further embodiment the binding domain-immunoglobulin fusion protein comprises a human IgE CH4 constant region polypeptide. In another further embodiment the antigen is a tumor antigen.

[0032] In certain other embodiments the present invention provides a binding domain-immunoglobulin fusion protein, comprising (a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein the binding domain polypeptide is capable of specifically binding to at least one antigen that is present on an immune effector cell and wherein the hinge region polypeptide comprises a polypeptide selected from the group consisting of a human IgA hinge region polypeptide, a human IgG hinge region polypeptide, and a human IgE hinge region polypeptide; (b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide, wherein said CH2 constant region polypeptide comprises a polypeptide selected from the group consisting of a human IgA CH2 constant region polypeptide, a human IgG CH2 constant region polypeptide, and a human IgE CH2 constant region polypeptide; (c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein said CH3 constant region polypeptide comprises a polypeptide selected from the group consisting of a human IgA CH3 constant region polypeptide, a human IgG CH3 constant region polypeptide, and a human IgE CH3 constant region polypeptide; and (d) a plasma membrane anchor domain polypeptide. In a further embodiment the membrane anchor domain polypeptide comprises a transmembrane domain polypeptide. In another further embodiment the membrane anchor domain polypeptide comprises a transmembrane domain polypeptide and a cytoplasmic tail polypeptide. In a still further embodiment the cytoplasmic tail polypeptide comprises an apoptosis signaling polypeptide sequence, which in a still further embodiment is derived from a receptor death domain polypeptide. In a further embodiment the death domain polypeptide comprises a polypeptide selected from an ITIM domain, an ITAM domain, FADD, TRADD, RAIDD, CD95 (FAS/Apo-1), TNFR1 or DR5. In another embodiment the apoptosis signaling polypeptide sequence comprises a polypeptide sequence derived from a caspase polypeptide that is caspase-3 or caspase-8. In another embodiment the plasma membrane anchor domain polypeptide comprises a glycosyl-phosphatidylinositol-linkage polypeptide sequence. In another embodiment the antigen that is present on an immune effector cell is CD2, CD28, CD30, CD40, CD50 (ICAM3), CD54 (ICAM1), CD80, CD86, B7-H1, CD134 (OX40), CD137 (41BB), CD152 (CTLA-4), CD153 (CD30 ligand), CD154 (CD40 ligand), ICOS, CD19, CD20, CD22, CD37, L6, CD3, CD4, CD25, CD8, CD11b, CD14, CD25, CD56 or CD69. In another embodiment the human IgG is human IgG1.

[0033] The invention provides, in another embodiment, a binding domain-immunoglobulin fusion protein, comprising (a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein the binding domain polypeptide is capable of specifically binding to at least one antigen that is present on a cancer cell surface and wherein the hinge region polypeptide comprises a polypeptide selected from the group consisting of a human IgA hinge region polypeptide, a human IgG hinge region polypeptide, and a human IgE hinge region polypeptide; (b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide, wherein the CH2 constant region polypeptide comprises a polypeptide that is a human IgA CH2 constant region polypeptide, a human IgG CH2 constant region polypeptide, or a human IgE CH2 constant region polypeptide; (c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein the CH3 constant region polypeptide comprises a polypeptide that is a human IgA CH3 constant region polypeptide, a human IgG CH3 constant region polypeptide, or a human IgE CH3 constant region polypeptide; and (d) a plasma membrane anchor domain polypeptide. In a further embodiment the membrane anchor domain polypeptide comprises a transmembrane domain polypeptide. In another embodiment the membrane anchor domain polypeptide comprises a transmembrane domain polypeptide and a cytoplasmic tail polypeptide. In another embodiment the membrane anchor domain polypeptide comprises a glycosyl-phosphatidylinositol-linkage polypeptide sequence. In another embodiment the human IgG is human IgG1.

[0034] In another embodiment the present invention provides a binding domain-immunoglobulin fusion protein, comprising (a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein said hinge region polypeptide comprises a wild-type human IgA hinge region polypeptide; (b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide, wherein said CH2 constant region polypeptide comprises a human IgA CH2 constant region polypeptide; and (c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein the CH3 constant region polypeptide comprises a polypeptide that is (i) a wild-type human IgA CH3 constant region polypeptide or (ii) a mutated human IgA CH3 constant region polypeptide that is incapable of associating with a J chain, wherein (1) the binding domain-immunoglobulin fusion protein is capable of at least one immunological activity selected from the group consisting of antibody dependent cell-mediated cytotoxicity and complement fixation, and (2) the binding domain polypeptide is capable of specifically binding to an antigen. In certain further embodiments the mutated human

IgA CH3 constant region polypeptide that is incapable of associating with a J chain is (i) a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:______ or (ii) a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:______.

[0035] In certain other embodiments the present invention provides a binding domain-immunoglobulin fusion protein, comprising (a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide; (b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide, wherein the CH2 constant region polypeptide comprises a llama CH2 constant region polypeptide that is a llama IgG1 CH2 constant region polypeptide, a llama IgG2 CH2 constant region polypeptide or a llama IgG3 CH2 constant region polypeptide; and (c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein said CH3 constant region polypeptide comprises a llama CH3 constant region polypeptide that is selected from the group consisting of a llama IgG1 CH3 constant region polypeptide, a llama IgG2 CH3 constant region polypeptide and a llama IgG3 CH3 constant region polypeptide wherein (1) the binding domain-immunoglobulin fusion protein is capable of at least one immunological activity selected from the group consisting of antibody dependent cell-mediated cytotoxicity and induction fixation of complement, and (2) the binding domain polypeptide is capable of specifically binding to an antigen. In a further embodiment the immunoglobulin hinge region polypeptide, the llama CH2 constant region polypeptide and the llama CH3 constant region polypeptide comprise sequences derived from a llama IgG1 polypeptide and the fusion protein does not include a llama IgG1 CH1 domain. In certain embodiments the invention provides any of the above described binding domain-immunoglobulin fusion proteins wherein the hinge region polypeptide is mutated to contain a glycosylation site, which in certain further embodiments is an asparagine-linked glycosylation site, an O-linked glycosylation site, a C-mannosylation site, a glypiation site or a phosphoglycation site. In certain embodiments the invention provides any of the above described binding domain-immunoglobulin fusion proteins wherein the binding domain polypeptide comprises two or more binding domain polypeptide sequences wherein each of the binding domain polypeptide sequences is capable of specifically binding to an antigen.

[0036] The present invention also provides, in certain embodiments, a binding domain-immunoglubulin fusion protein, comprising (a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein the hinge region polypeptide comprises an alternative hinge region polypeptide sequence; (b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide; and (c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein: (1) the binding domain-immunoglobulin fusion protein is capable of at least one immunological activity selected from the group consisting of antibody dependent cell-mediated cytotoxicity and complement fixation, and (2) the binding domain polypeptide is capable of specifically binding to an antigen.

[0037] Turning to another embodiment there is provided a binding domain-immunoglobulin fusion protein, comprising (a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein the binding domain polypeptide is capable of specifically binding to at least one antigen that is present on a cancer cell surface and wherein the hinge region polypeptide comprises an alternative hinge region polypeptide sequence; (b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide, wherein said CH2 constant region polypeptide comprises a polypeptide selected from the group consisting of a human IgA CH2 constant region polypeptide, a human IgG CH2 constant region polypeptide, and a human IgE CH2 constant region polypeptide; (c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein the CH3 constant region polypeptide comprises a polypeptide that is a human IgA CH3 constant region polypeptide, a human IgG CH3 constant region polypeptide, or a human IgE CH3 constant region polypeptide; and (d) a plasma membrane anchor domain polypeptide. In certain further embodiments the alternative hinge region polypeptide sequence comprises a polypeptide sequence of at least ten continuous amino acids that are present in a sequence selected from SEQ ID NOS:___-___.

[0038] In certain embodiments the present invention provides an isolated polynucleotide encoding any one of the above described binding domain-immunoglobulin fusion proteins, and in other embodiments the invention provides a recombinant expression construct comprising any such polynucleotide that is operably linked to a promoter. In other embodiments there is provided a host cell transformed or transfected with any such recombinant expression construct. In a related embodiment there is provided a method of producing a binding domain-immunoglobulin fusion protein, comprising the steps of (a) culturing a host cell as just described under conditions that permit expression of the binding domain-immunoglobulin fusion protein; and (b) isolating the binding domain-immunoglobulin fusion protein from the host cell culture. In another embodiment there is provided a pharmaceutical composition comprising any one of the above described binding domain-immunoglobulin fusion proteins in combination with a physiologically acceptable carrier. In another embodiment the invention provides a pharmaceutical composition comprising an isolated polynucleotide encoding any one of the above described binding domain-immunoglobulin fusion proteins, in combination with a physiologically acceptable carrier. In another embodiment the invention provides a method of treating a subject having or suspected of having a malignant condition or a B-cell disorder, comprising administering to a patient a therapeutically effective amount of any of the pharmaceutical compositions just described. In certain further embodiments the malignant condition or B-cell disorder is a B-cell lymphoma or a disease characterized by autoantibody production, and in certain other further embodiments the malignant condition or B-cell disorder is rheumatoid arthritis, myasthenia gravis, Grave's disease, type I diabetes mellitus, multiple sclerosis or an autoimmune disease. In certain other embodiments the malignant condition is melanoma, carcinoma or sarcoma.

[0039] It is another aspect of the present invention to provide a binding domain-immunoglobulin fusion protein,

comprising (a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein said hinge region polypeptide is selected from the group consisting of (i) a mutated hinge region polypeptide that contains no cysteine residues and that is derived from a wild-type immunoglobulin hinge region polypeptide having one or more cysteine residues, (ii) a mutated hinge region polypeptide that contains one cysteine residue and that is derived from a wild-type immunoglobulin hinge region polypeptide having two or more cysteine residues, (iii) a wild-type human IgA hinge region polypeptide, (iv) a mutated human IgA hinge region polypeptide that contains no cysteine residues and that is derived from a wild-type human IgA region polypeptide, and (v) a mutated human IgA hinge region polypeptide that contains one cysteine residue and that is derived from a wild-type human IgA region polypeptide; (b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide; and (c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein: (1) the binding domain-immunoglobulin fusion protein is capable of at least one immunological activity selected from the group consisting of antibody dependent cell-mediated cytotoxicity and complement fixation, and (2) the binding domain polypeptide is capable of specifically binding to an antigen. In one embodiment the immunoglobulin hinge region polypeptide is a mutated hinge region polypeptide and exhibits a reduced ability to dimerize, relative to a wild-type human immunoglobulin G hinge region polypeptide. In another embodiment the binding domain polypeptide comprises at least one immunoglobulin variable region polypeptide that is an immunoglobulin light chain variable region polypeptide or an immunoglobulin heavy chain variable region polypeptide. In a further embodiment the immunoglobulin variable region polypeptide is derived from a human immunoglobulin.

[0040] In another embodiment the binding domain Fv-immunoglobulin fusion protein binding domain polypeptide comprises (a) at least one immunoglobulin light chain variable region polypeptide; (b) at least one immunoglobulin heavy chain variable region polypeptide; and (c) at least one linker peptide that is fused to the polypeptide of (a) and to the polypeptide of (b). In a further embodiment the immunoglobulin light chain variable region and heavy chain variable region polypeptides are derived from human immunoglobulins.

[0041] In another embodiment at least one of the immunoglobulin heavy chain CH2 constant region polypeptide and the immunoglobulin heavy chain CH3 constant region polypeptide is derived from a human immunoglobulin heavy chain. In another embodiment the immunoglobulin heavy chain constant region CH2 and CH3 polypeptides are of an isotype selected from human IgG and human IgA. In another embodiment the antigen is selected from the group consisting of CD19, CD20, CD37, CD40 and L6. In certain further embodiments of the above described fusion protein, the linker polypeptide comprises at least one polypeptide having as an amino acid sequence Gly-Gly-Gly-Gly-Ser [SEQ ID NO:______], and in certain other embodiments the linker polypeptide comprises at least three repeats of a polypeptide having as an amino acid sequence Gly-Gly-Gly-Gly-Ser [SEQ ID NO:______]. In certain embodiments the immunoglobulin hinge region polypeptide comprises a human IgA hinge region polypeptide. In certain embodiments the binding domain polypeptide comprises a CD154 extracellular domain. In certain embodiments the binding domain polypeptide comprises a CD154 extracellular domain and at least one immunoglobulin variable region polypeptide.

[0042] In other embodiments the invention provides an isolated polynucleotide encoding any of the above described binding domain-immunoglobulin fusion proteins, and in related embodiments the invention provides a recombinant expression construct comprising such a polynucleotide, and in certain further embodiments the invention provides a host cell transformed or transfected with suc a recombinant expression construct. In another embodiment the invention provides a method of producing a binding domain-immunoglobulin fusion protein, comprising the steps of (a) culturing the host cell as just described, under conditions that permit expression of the binding domain-immunoglobulin fusion protein; and (b) isolating the binding domain-immunoglobulin fusion protein from the host cell culture.

[0043] The present invention also provides in certain embodiments a pharmaceutical composition comprising a binding domain-immunoglobulin fusion protein as described above, in combination with a physiologically acceptable carrier. In another embodiment there is provided a method of treating a subject having or suspected of having a malignant condition or a B-cell disorder, comprising administering to a patient a therapeutically effective amount of an above described binding domain-immunoglobulin fusion protein. In certain further embodiments the malignant condition or B-cell disorder is a B-cell lymphoma or a disease characterized by autoantibody production, and in certain other further embodiments the malignant condition or B-cell disorder is rheumatoid arthritis, myasthenia gravis, Grave's disease, type I diabetes mellitus, multiple sclerosis or an autoimmune disease.

[0044] These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entireties as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS

[0045] FIG. 1 shows DNA and deduced amino acid sequences [SEQ ID NOS:______] of 2H7scFv-Ig, a binding domain-immunoglobulin fusion protein capable of specifically binding CD20.

[0046] FIG. 2 shows production levels of 2H7 scFv-Ig by transfected, stable CHO lines and generation of a standard curve by binding of purified 2H7 scFv-Ig to CHO cells expressing CD20.

[0047] FIG. 3 shows SDS-PAGE analysis of multiple preparations of isolated 2H7scFv-Ig protein.

[0048] FIG. 4 shows complement fixation (FIG. 4A) and mediation of antibody-dependent cellular cytotoxicity (ADCC, FIG. 4B)) by 2H7scFv-Ig.

[0049] FIG. 5 shows the effect of simultaneous ligation of CD20 and CD40 on growth of normal B cells.

[0050] FIG. 6 shows the effect of simultaneous ligation of CD20 and CD40 on CD95 expression and induction of apoptosis in a B lymphoblastoid cell line.

[0051] FIG. 7 shows DNA and deduced amino acid sequences [SEQ ID NOS:______] of 2H7scFv-CD154 L2 (FIG. 7A, SEQ ID NOS:______) and 2H7scFv-CD154 S4 (FIG. 7B, SEQ ID NOS:______) binding domain-immunoglobulin fusion proteins capable of specifically binding CD20 and CD40.

[0052] FIG. 8 shows binding of 2H7scFv-CD154 binding domain-immunoglobulin fusion proteins to CD20+ CHO cells by flow immunocytofluorimetry.

[0053] FIG. 9 shows binding of Annexin V to B cell lines Ramos, BJAB, and T51 after binding of 2H7scFv-CD154 binding domain-immunoglobulin fusion protein to cells.

[0054] FIG. 10 shows effects on proliferation of B cell line T51 following binding of 2H7scFv-CD154 binding domain-immunoglobulin fusion protein.

[0055] FIG. 11 depicts schematic representations of the structures of 2H7ScFv-Ig fusion proteins [SEQ ID NOS:______] referred to as CytoxB or CytoxB derivatives: CytoxB-MHWTG1C (2H7 ScFv, mutant hinge, wild-type human IgG1 Fc domain), CytoxB-MHMG1C (2H7 ScFv, mutant hinge, mutated human IgG1 Fc domain) and CytoxB-IgAHWTHG1C (2H7 ScFv, human IgA-derived hinge [SEQ ID NO:______], wild-type human IgG1 Fc domain). Arrows indicate position numbers of amino acid residues believed to contribute to FcR binding and ADCC activity (heavy arrows), and to complement fixation (light arrows). Note absence of interchain disulfide bonds.

[0056] FIG. 12 shows SDS-PAGE analysis of isolated CytoxB and 2H7scFv-CD154 binding domain-immunoglobulin fusion proteins.

[0057] FIG. 13 shows antibody dependent cell-mediated cytotoxicity (ADCC) activity of CytoxB derivatives.

[0058] FIG. 14 shows complement dependent cytotoxicity (CDC) of CytoxB derivatives.

[0059] FIG. 15 shows serum half-life determinations of CytoxB-MHWTG1C in macaque blood samples.

[0060] FIG. 16 shows effects of CytoxB-MHWTG1C on levels of circulating CD40+ B cells in macaque blood samples.

[0061] FIG. 17 shows production levels of HD37 (CD19-specific) ScFv-Ig by transfected mammalian cell lines and generation of a standard curve by binding of purified HD37 ScFv-Ig to cells expressing CD19.

[0062] FIG. 18 shows production levels of L6 (carcinoma antigen) ScFv-Ig by transfected, stable CHO lines and generation of a standard curve by binding of purified L6 ScFv-Ig to cells expressing L6 antigen.

[0063] FIG. 19 shows ADCC activity of binding domain-immunoglobulin fusion proteins 2H7 ScFv-Ig, HD37 ScFv-Ig and G28-1 (CD37-specific) ScFv-Ig.

[0064] FIG. 20 shows ADCC activity of L6 ScFv-Ig fusion proteins.

[0065] FIG. 21 shows SDS-PAGE analysis of L6 ScFv-Ig and 2H7 ScFv-Ig fusion proteins.

[0066] FIG. 22 shows SDS-PAGE analysis of G28-1 ScFv-Ig and HD37 ScFv-Ig fusion proteins.

[0067] FIG. 23 presents a sequence alignment of immunoglobulin hinge and CH2 domains of human IgG1 (SEQ ID NO:______) with the hinge and CH2 domains of llama IgG1 (SEQ ID NO:______, IgG2 (SEQ ID NO:______), and IgG3 (SEQ ID NO:______).

[0068] FIG. 24 illustrates migration of purified 2H7 scFv llama IgG fusion proteins in a 10% SDS polyacrylamide gel. Purified fusion proteins (5 μg per sample) were prepared in non-reducing sample buffer (lanes 2-5) and in reducing sample buffer (lanes 6-9). Lane 1: molecular weight markers (non-reduced); lanes 2 and 6: 2H7 scFv-llama IgG1 (SEQ ID NO:______); Lanes 3 and 7: 2H7 scFv-llama IgG2 (SEQ ID NO:______): lanes 4 and 8: 2H7 scFv-llama IgG3 (SEQ ID NO:______); and Lanes 5 and 9: Rituximab (chimeric anti-CD20 antibody (human IgG1 constant region)).

[0069] FIG. 25 shows binding of 2H7 scFv-llama IgG1 (SEQ ID NO:______), 2H7 scFv-llama IgG2 (SEQ ID NO:______), and 2H7 scFv-llama IgG3 (SEQ ID NO:______) to CD20+ CHO cells detected by flow immunocytofluorimetry.

[0070] FIG. 26 depicts CDC activity of 2H7 scFv llama IgG fusion proteins, 2H7 scFv-llama IgG1 (SEQ ID NO:______), 2H7 scFv-llama IgG2 (SEQ ID NO:______), and 2H7 scFv-llama IgG3 (SEQ ID NO:______), and 2H7 scFv human IgG1 (2H7 scFv IgG WTH WTCH2CH3) (SEQ ID NO:______) against BJAB cells in the presence of rabbit complement. Rituximab was included as a positive control.

[0071] FIG. 27 shows ADCC activity of 2H7 scFv llama IgG fusion proteins, 2H7 scFv-llama IgG1 (SEQ ID NO:______), 2H7 scFv-llama IgG2 (SEQ ID NO:______), and 2H7 scFv-llama IgG3 (SEQ ID NO:______). Effector cells (human PBMC) were combined with target cells (BJAB cells) at three different ratios, 1:25, 1:50, and 1:100. Rituximab was included as a positive control. Each data point represents three separate measurements.

[0072] FIG. 28 shows ADCC activity of 2H7 scFv llama IgG fusion proteins, 2H7 scFv-llama IgG1 (SEQ ID NO:______), 2H7 scFv-llama IgG2 (SEQ ID NO:______), and 2H7 scFv-llama IgG3 (SEQ ID NO:______). Effector cells (llama PBMC) were combined with target cells (BJAB cells) at three different ratios, 1:25, 1:50, and 1:100. Rituximab was included as a positive control. Each data point represents three separate measurements.

[0073] FIG. 29 depicts CDC activity of Reh cells (acute lymphocytic leukemia) expressing scFv-Ig fusion proteins on the cell surface. Reh cells were transfected with constructs encoding scFv antibodies specific for human costimulatory molecules, CD152, CD28, CD40, and CD20, fused to human IgG1 wild-type hinge-CH2-CH3, which was fused to human CD80 transmembrane and cytoplasmic tail domains. CDC activity was measured in the presence and absence of rabbit complement (plus C' and no C', respectively). The data represent the average of duplicate samples. Reh anti-hCD152 scFvIg: Reh cells transfected with polynucleotide 10A8 scFv IgG MTH (SSS) MT CH2CH3 (SEQ ID NO:______); Reh anti-hCD28scFvIg: 2E12 scFv IgG MTH (SSS) MT CH2CH3 (SEQ ID NO:______); Reh anti-hCD40scFvIg: 4.2.220 scFv IgG MTH (SSS) MT CH2CH3 (SEQ ID NO:______); and Reh anti-hCD20scFvIg: 2H7 scFv IgG MTH (SSS) MT CH2CH3 (SEQ ID NO:______).

[0074] FIG. 30 presents ADCC activity of Reh cells that were transfected with constructs encoding scFv antibodies specific for human costimulatory molecules, CD152, CD28, CD40, and CD20, as described for FIG. 29, and for murine CD3, fused to human mutant IgG1 hinge and mutant CH2 and wild type CH3 (Reh anti-mCD3scFv designating Reh cells transfected with polynucleotide 500A2 scFv IgG MTH (SSS) MTCH2WTCH3 SEQ ID NO:______), which was fused to human CD80 transmembrane and cytoplasmic tail domains. The data represent the average of quadruplicate samples.

[0075] FIG. 31 lists immunoglobulin constant region constructs that were used in experiments illustrated in subsequent figures.

[0076] FIG. 32 depicts CDC activity of CTLA-4 Ig fusion proteins, CTLA-4 IgG WTH (CCC) WTCH2CH3 (SEQ ID NO:______) (2 μg/ml) and CTLA-4 IgG MTH MTCH2WTCH3 (SEQ ID NO:______) (2 μg/ml), in the presence and absence of rabbit complement (plus C' and no C', respectively). The target cells were Reh cells and Reh cells transfected with CD80 (Reh CD80.10).

[0077] FIG. 33 shows ADCC activity of CTLA-4 Ig fusion proteins, CTLA-4 IgG WTH (CCC) WTCH2CH3 (SEQ ID NO:______) (2 μg/ml) and CTLA-4 IgG MTH MTCH2WTCH3 (SEQ ID NO:______) (2 μg/ml). Effector cells, human PBMC, were added to target cells, Reh or Reh CD80.1, at the ratios indicated. FIG. 33A presents the level of natural killing in Reh CD80.1 cells in the absence of any Ig fusion protein. FIG. 33B presents ADCC mediated by CTLA-4 IgG MTH MTCH2WTCH3, and FIG. 33C presents ADCC mediated by CTLA-4 IgG WTH (CCC) WTCH2CH3. Each data point represents the average percent specific killing measured in four sample wells.

[0078] FIG. 34 illustrates binding of 2H7 (anti-CD20) scFv Ig fusion proteins to (CD20+) CHO cells by flow immunocytofluorimetry.

[0079] FIG. 35 presents an immunoblot of 2H7 scFv IgG and IgA fusion proteins. COS cells were transiently transfected with various 2H7 scFv Ig fusion protein constructs. The expressed polypeptides were immune precipitated with protein A, separated in a non-reducing SDS polyacrylamide gel, and then transferred to a polyvinyl fluoride membrane. Proteins were detected using an anti-human IgG (Fc specific) horseradish peroxidase conjugate. Lane 1: vector only; lane 2: 2H7 scFv IgG WTH (CCC) WTCH2CH3 (SEQ ID NO:______); lane 3: 2H7 scFv IgG MTH (CSS) WTCH2CH3 (SEQ ID NO:______); lane 4: 2H7 scFv IgG MTH (SCS) WTCH2CH3 (SEQ ID NO:______); lane 5: 2H7 scFv IgAH IgG WTCH2CH3 (SEQ ID NO:______); and lane 6: 2H7 scFv IgG MTH (SSS) WTCH2CH3 (SEQ ID NO:______).

[0080] FIG. 36 illustrates binding of 2H7 scFv IgAH IgACH2CH3 polypeptide (SEQ ID NO:______) and 2H7 scFv IgAH IgAT4 (SEQ ID NO:______) to (CD20+) CHO cells by flow immunocytofluorimetry. The source of the polypeptides was culture supernatants from transiently transfected COS cells. COS cells transfected with a plasmid comprising a sequence encoding 2H7 scFv IgAH IgACH2CH3 were co-transfected with a plasmid containing nucleotide sequence encoding human J chain.

[0081] FIG. 37 illustrates ADCC activity of anti-CD20 (2H7) scFv Ig fusion proteins against BJAB target cells using whole blood as the source of effector cells. Purified 2H7 scFv Ig fusion proteins were titrated and combined with ^{51}Cr-labeled BJAB cells (5×10^4) and whole blood (1:4 final dilution). Each data point represents the average percent specific killing measured in four sample wells.

[0082] FIG. 38 demonstrates ADCC activity of 2H7 scFv Ig fusion proteins (5 μg/ml) against ^{51}Cr-labeled BJAB cells at 0.25, 0.125, and 0.625 dilutions of whole blood. Each data point represents the average percent specific killing measured in four sample wells.

[0083] FIG. 39 shows a comparison of ADCC activity of 2H7 scFv IgG MTH (SSS) WTCH2CH3 (5 μg/ml) and 2H7 scFv IgAH IgACH2CH3 (5 μg/ml) when human PBMC are the source of effector cells (FIG. 39A) and when human whole blood is the source of effector cells (FIG. 39B).

[0084] FIG. 40 presents an immunoblot of 2H7 scFv IgG fusion proteins. COS cells were transiently transfected with various 2H7 scFv Ig fusion protein constructs. Culture supernatants containing the expressed polypeptides were separated in a non-reducing SDS polyacrylamide gel, and then were transferred to a polyvinyl fluoride membrane. Proteins were detected using an anti-human IgG (Fc specific) horseradish peroxidase conjugate. Lanes 1-5: purified 2H7 scFv IgG MTH (SSS) WTCH2CH3 at 40 ng, 20 ng, 10 ng/ 5 ng, and 2.5 ng per lane, respectively. Culture supernatants were separated in lanes 6-9. Lane 6: 2H7 scFv IgG WTH (CCC) WTCH2CH3; lane 7: 2H7 scFv IgG MTH (CSS) WTCH2CH3; lane 8: 2H7 scFv IgG MTH (SCS) WTCH2CH3; and lane 9: 2H7 scFv VHSER11 IgG MTH (SSS) WTCH2CH3. The molecular weight (kDal) of marker proteins is indicated on the left side of the immunoblot.

[0085] FIG. 41A illustrates cell surface expression of 1D8 (anti-murine 4-1BB) scFv IgG WTH WTCH2CH3-CD80 fusion protein on K1735 melanoma cells by flow immunofluorimetry. The scFv fusion protein was detected with phycoerythrin-conjugated $F(ab')_2$ goat anti-human IgG. FIG. 41B depicts growth of tumors in naïve C3H mice transplanted by subcutaneous injection with wild type K1735 melanoma cells (K1735-WT) or with K1735 cells transfected with 1D8 scFv IgG WTH WTCH2CH3-CD80 (K1735-1D8). Tumor growth was monitored by measuring the size of the tumor. FIG. 41C demonstrates the kinetics of tumor growth in naive C3H mice injected intraperitoneally with monoclonal antibodies to remove $CD8^+$, $CD4^+$, or both $CD4^+$ and $CD8^+$ T cells prior to transplantation of the animals with K1735-1D8 cells.

[0086] FIG. 42 demonstrates therapy of established K1735-WT tumors using K1735-1D8 as an immunogen. Six days after mice were transplanted with K1735-WT tumors, one group (five animals) was injected subcutaneously with K1735-1D8 cells (open circles) or irradiated K1735-WT cells (solid squares) on the contralateral side. A control group of mice received PBS (open squares). Treatments were repeated on the days indicated by the arrows.

[0087] FIG. 43 shows the growth of tumors in animals that were injected subcutaneously with 2×10^6 K1735-WT cells (solid squares) and the growth of tumors in animals that were injected subcutaneously with 2×10^6 K1735-WT cells plus 2×10^5 K1735-1D8 cells (open triangles).

[0088] FIG. 44 presents a flow cytometry analysis of Ag104 murine sarcoma tumor cells transfected with 1D8

scFv IgG WTH WTCH2CH3-CD80 isolated after repeated rounds of panning against anti-human IgG. Transfected cells expressing 1D8 scFv IgG WTH WTCH2CH3-CD80 were detected with fluoroisothiocyanate (FITC)-conjugated goat anti-human IgG (depicted in black). Untransfected cells are shown in gray.

[0089] **FIG. 45** illustrates migration of various 2H7 scFv Ig fusion proteins in a 10% SDS-PAGE gel. 2H7 was the anti-CD20 scFv and [220] was the anti-CD40 scFv. Lane 1: Bio-Rad prestained molecular weight standards; lane 2: anti-CD20 scFv IgG MTH (SSS) MTCH2WTCH3; lane 3: anti-CD20 scFv IgG MTH (SSS) WTCH2CH3; lane 4: 2H7 scFv IgAH IgG WTCH2CH3; lane 5: anti-CD20-anti-CD40 scFv IgG MTH (SSS) MTCH2WTCH3; lane 6: Rituximab; lane 7: Novex Multimark® molecular weight standards.

[0090] **FIG. 46** illustrates effector function as measured in an ADCC assay of 2H7 Ig fusion proteins that contain a mutant CH2 domain or wild type CH2 domain. The percent specific killing of BJAB target cells in the presence of human PBMC effector cells by 2H7 scFv IgG MTH (SSS) MTCH2WTCH3 (diamonds) was compared to 2H7 scFv IgG MTH (SSS) WTCH2CH3 (squares) and 2H7 scFv IgAH IgG WTCH2CH3 (triangles) and Rituximab (circles).

[0091] **FIG. 47** shows cell surface expression of an anti-human CD3 scFv IgG WTH WTCH2CH3-CD80 (SEQ ID NO:______) fusion protein on Reh cells (**FIG. 47A**) and T51 lymphoblastoid cells (**FIG. 47B**) by flow immunocytofluorimetry.

[0092] **FIG. 48** presents the percent specific killing of untransfected Reh and T51 cells and the percent specific killing of Reh cells (Reh anti-hCD3) (**FIG. 48A**) and T51 cells (T51 anti-hCD3) (**FIG. 48B**) that were transfected with a construct encoding scFv antibodies specific for human CD3, fused to human IgG1 wild-type hinge-CH2-CH3, which was fused to human CD80 transmembrane and cytoplasmic tail domains (anti-human CD3 scFv IgG WTH WTCH2CH3-CD80 (SEQ ID NO:______). Human PBMC (effector cells) were combined with BJAB target cells at the ratios indicated.

[0093] **FIG. 49** illustrates binding of 5B9, an anti-murine CD137 (4-1BB) monoclonal antibody, and a 5B9 scFv IgG fusion protein (5B9 scFv IgG MTH (SSS) WTCH2CH3 (SEQ ID NO:______)) to stimulated human PBMC. Binding of the 5B9 scFv IgG fusion protein was detected by flow immunocytofluorimetry using FITC conjugated goat anti-human IgG. Binding of the 5B9 monoclonal antibody was detected with FITC conjugated goat anti-mouse IgG.

DETAILED DESCRIPTION OF THE INVENTION

[0094] The present invention is directed to binding domain-immunoglobulin fusion proteins and to related compositions and methods, which will be useful in immunotherapeutic and immunodiagnostic applications, and which offer certain advantages over antigen-specific polypeptides of the prior art. The fusion proteins of the present invention are preferably single polypeptide chains that comprise, in pertinent part, the following fused domains: a binding domain polypeptide, an immunoglobulin hinge region polypeptide, an immunoglobulin heavy chain CH2 constant region polypeptide, and an immunoglobulin heavy chain CH3 constant region polypeptide. According to certain preferred embodiments the fusion proteins of the present invention further comprise a plasma membrane anchor domain. According to certain other preferred embodiments the fusion proteins of the present invention further comprise an immunoglobulin heavy chain CH4 constant region polypeptide. In particularly preferred embodiments, the polypeptide domains of which the binding domain-immunoglobulin fusion protein is comprised are, or are derived from, polypeptides that are the products of human gene sequences, but the invention need not be so limited and may in fact relate to binding domain-immunoglobulin fusion proteins as provided herein that are derived from any natural or artificial source, including genetically engineered and/or mutated polypeptides.

[0095] The present invention relates in part to the surprising observation that the binding domain-immunoglobulin fusion proteins described herein are capable of immunological activity. More specifically, these proteins retain the ability to participate in well known immunological effector activities including antibody dependent cell mediated cytotoxicity (ADCC, e.g., subsequent to antigen binding on a cell surface, engagement and induction of cytotoxic effector cells bearing appropriate Fc receptors, such as natural killer (NK) cells bearing FcRγIII, under appropriate conditions;) and/or complement fixation in complement dependent cytotoxicity (CDC, e.g., subsequent to antigen binding on a cell surface, recruitment and activation of cytolytic proteins that are components of the blood complement cascade; for reviews of ADCC and CDC see, e.g., Carter, 2001 *Nat. Rev. Canc.* 1:118; Sulica et al., 2001 *Int. Rev. Immunol.* 20:371; Maloney et al., 2002 *Semin. Oncol.* 29:2; Sondel et al., 2001; Maloney 2001 *Anticanc. Drugs* 12 Suppl.2:1-4; IgA activation of complement by the alternative pathway is described, for example, in Schneiderman et al., 1990 *J. Immunol.* 145:233), despite having structures that would not be expected to be capable of promoting such effector activities. As described in greater detail below, ADCC and CDC are unexpected functions for fusion proteins comprising immunoglobulin heavy chain regions and having the structures described herein, and in particular for immunoglobulin fusion proteins comprising immunoglobulin hinge region polypeptides that are compromised in their ability to form interchain, homodimeric disulfide bonds.

[0096] Another advantage afforded by the present invention is a binding domain-immunoglobulin fusion polypeptide that can be produced in substantial quantities that are typically greater than those routinely attained with single-chain antibody constructs of the prior art. In preferred embodiments, the binding domain-immunoglobulin fusion polypeptides of the present invention are recombinantly expressed in mammalian expression systems, which offer the advantage of providing polypeptides that are stable in vivo (e.g., under physiological conditions). According to non-limiting theory, such stability may derive in part from posttranslational modifications, and specifically glycosylation, of the fusion proteins. Production of the present binding domain-immunoglobulin fusion proteins via recombinant mammalian expression has been attained in static cell cultures at a level of greater than 50 mg protein per liter culture supernatant and has been routinely observed in such cultures at 10-50 mg/l, such that preferably at least 10-50 mg/l may be produced under static culture conditions; also contemplated are enhanced production of the fusion proteins

using art-accepted scale-up methodologies such as "fed batch" (i.e., non-static) production, where yields of at least 5-500 mg/l, and in some instances at least 0.5-1 gm/l, depending on the particular protein product, are obtained.

[0097] A binding domain polypeptide according to the present invention may be any polypeptide that possesses the ability to specifically recognize and bind to a cognate biological molecule or complex of more than one molecule or assembly or aggregate, whether stable or transient, of such a molecule, which includes a protein, polypeptide, peptide, amino acid, or derivative thereof; a lipid, fatty acid or the like, or derivative thereof; a carbohydrate, saccharide or the like or derivative thereof, a nucleic acid, nucleotide, nucleoside, purine, pyrimidine or related molecule, or derivative thereof, or the like; or any combination thereof such as, for example, a glycoprotein, a glycopeptide, a glycolipid, a lipoprotein, a proteolipid; or any other biological molecule that may be present in a biological sample. Biological samples may be provided by obtaining a blood sample, biopsy specimen, tissue explant, organ culture, biological fluid or any other tissue or cell preparation from a subject or a biological source. The subject or biological source may be a human or non-human animal, a primary cell culture or culture adapted cell line including but not limited to genetically engineered cell lines that may contain chromosomally integrated or episomal recombinant nucleic acid sequences, immortalized or immortalizable cell lines, somatic cell hybrid cell lines, differentiated or differentiatable cell lines, transformed cell lines and the like. In certain preferred embodiments of the invention, the subject or biological source may be suspected of having or being at risk for having a malignant condition or a B-cell disorder as provided herein, which in certain further preferred embodiments may be an autoimmune disease, and in certain other preferred embodiments of the invention the subject or biological source may be known to be free of a risk or presence of such disease.

[0098] A binding domain polypeptide may therefore be any naturally occurring or recombinantly produced binding partner for a cognate biological molecule as provided herein that is a target structure of interest, herein referred to as an "antigen" but intended according to the present disclosure to encompass any target biological molecule to which it is desirable to have the subject invention fusion protein specifically bind. Binding domain-immunoglobulin fusion proteins are defined to be "immunospecific" or capable of specifically binding if they bind a desired target molecule such as an antigen as provided herein, with a K_a of greater than or equal to about 10^4 M^{-1}, preferably of greater than or equal to about 10^5 M^{-1}, more preferably of greater than or equal to about 10^6 M^{-1} and still more preferably of greater than or equal to about 10^7 M^{-1}. Affinities of binding domain-immunoglobulin fusion proteins according to the present invention can be readily determined using conventional techniques, for example those described by Scatchard et al., *Ann. N.Y. Acad. Sci.* 51:660 (1949). Such determination of fusion protein binding to target antigens of interest can also be performed using any of a number of known methods for identifying and obtaining proteins that specifically interact with other proteins or polypeptides, for example, a yeast two-hybrid screening system such as that described in U.S. Pat. No. 5,283,173 and U.S. Pat. No. 5,468,614, or the equivalent.

[0099] Preferred embodiments of the subject invention binding domain-immunoglobulin fusion protein comprise binding domains that include at least one immunoglobulin variable region polypeptide, such as all or a portion or fragment of a heavy chain or a light chain V-region, provided it is capable of specifically binding an antigen or other desired target structure of interest as described herein. In other preferred embodiments the binding domain comprises a single chain immunoglobulin-derived Fv product, which may include all or a portion of at least one immunoglobulin light chain V-region and all or a portion of at least one immunoglobulin heavy chain V-region, and which further comprises a linker fused to the V-regions; preparation and testing such constructs are described in greater detail herein and are well known in the art. As described herein and as also known in the art, immunoglobulins comprise products of a gene family the members of which exhibit a high degree of sequence conservation, such that amino acid sequences of two or more immunoglobulins or immunoglobulin domains or regions or portions thereof (e.g., VH domains, VL domains, hinge regions, CH2 constant regions, CH3 constant regions) can be aligned and analyzed to identify portions of such sequences that correspond to one another, for instance, by exhibiting pronounced sequence homology. Determination of sequence homology may be readily determined with any of a number of sequence alignment and analysis tools, including computer algorithms well known to those of ordinary skill in the art, such as Align or the BLAST algorithm (Altschul, *J. Mol. Biol.* 219:555-565, 1991; Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915-10919, 1992), which is available at the NCBI website (http://www/ncbi.nlm.nih.gov/cgi-bin/BLAST). Default parameters may be used.

[0100] Portions of a particular immunoglobulin reference sequence and of any one or more additional immunoglobulin sequences of interest that may be compared to the reference sequence are regarded as "corresponding" sequences, regions, fragments or the like, based on the convention for numbering immunoglobulin amino acid positions according to Kabat, *Sequences of Proteins of Immunological Interest*, (5^{th} ed. Bethesda, Md.: Public Health Service, National Institutes of Health (1991)). For example, according to this convention, the immunoglobulin family to which an immunoglobulin sequence of interest belongs is determined based on conservation of variable region polypeptide sequence invariant amino acid residues, to identify a particular numbering system for the immunoglobulin family, and the sequence(s) of interest can then be aligned to assign sequence position numbers to the individual amino acids which comprise such sequence(s). Preferably at least 70%, more preferably at least 80%-85% or 86%-89%, and still more preferably at least 90%, 92%, 94%, 96%, 98% or 99% of the amino acids in a given amino acid sequence of at least 1000, more preferably 700-950, more preferably 350-700, still more preferably 100-350, still more preferably 80-100, 70-80, 60-70, 50-60, 40-50 or 30-40 consecutive amino acids of a sequence, are identical to the amino acids located at corresponding positions in a reference sequence such as those disclosed by Kabat (1991) or in a similar compendium of related immunoglobulin sequences, such as may be generated from public databases (e.g., Genbank, SwissProt, etc.) using sequence alignment tools as described above. In certain preferred embodiments, an immunoglobulin sequence of interest or a region, portion, derivative or

fragment thereof is greater than 95% identical to a corresponding reference sequence, and in certain preferred embodiments such a sequence of interest may differ from a corresponding reference at no more than 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 amino acid positions.

[0101] For example, in certain embodiments the present invention is directed to a binding domain-immunoglobulin fusion protein comprising in pertinent part a human immunoglobulin heavy chain variable region polypeptide comprising a mutation at an amino acid at a location corresponding to amino acid position 11 in SEQ ID NO:______, which comprises a murine VH-derived sequence, and regarding which it is noteworthy that at a relatively limited number of immunoglobulin VH sequence positions, including position 11, amino acid conservation is observed in the overwhelming majority of VH sequences analyzed across mammalian species lines (e.g., Leu11, Val37, Gly44, Leu45, Trp47; Nguyen et al., 1998 *J. Mol. Biol* 275:413). These amino acid side chains are located at the surface of the variable domain (V_H), where they may contact residues of the C_H1 (Leu11) and the V_L domains (Val37, Gly44, Leu45, and Trp47) and may, in the absence of light chains, contribute to stability and solubility of the protein (see, e.g., Chothia et al., 1985 *J. Mol. Biol.* 186:651; Muyldermans et al., 1994 *Prot. Engineer.* 7:1129; Desmyter et al., 1996 *Nat. Struct. Biol.* 3:803; Davies et al., 1994 *FEBS Lett.* 339:285). As another example, by reference to immunoglobulin sequence compendia and databases such as those cited above, the relatedness of two or more immunoglobulin sequences to each other can readily and without undue experimentation be established in a manner that permits identification of the animal species of origin, the class and subclass (e.g., isotype) of a particular immunoglobulin or immunoglobulin region polypeptide sequence. Any immunoglobulin variable region polypeptide sequence, including VH and/or VL and/or single-chain variable region (sFv) sequences or other V region-derived sequences or the like, may be used as a binding domain. Preferred embodiments include immunoglobulin V region polypeptide sequences derived from monoclonal antibodies such as murine or other rodent antibodies, or monoclonal antibodies derived from other sources such as goat, rabbit, equine, bovine, camelid or other species, including transgenic animals, and also including human or humanized monoclonal antibodies. Non-limiting examples include variable region polypeptide sequences derived from mAb such as those described in greater detail in the Examples below, for instance, CD20-specific murine monoclonal antibodies (e.g., 2H7), mAb L6 (specific for a carbohydrate-defined epitope and available from American Type Culture Collection, Manassas, Va., as hybridoma HB8677), and mAb specific for CD28 (e.g., mAb 2E12), CD40, CD80, CD137 (e.g., mAb 5B9 or mAb 1D8 which recognizes the murine homologue of CD137, 41BB) and CD152 (CTLA-4).

[0102] Other binding domain polypeptides may comprise any protein or portion thereof that retains the ability to specifically bind an antigen as provided herein, including non-immunoglobulins. Accordingly the invention contemplates fusion proteins comprising binding domain polypeptides that are derived from polypeptide ligands such as hormones, cytokines, chemokines, and the like; cell surface or soluble receptors for such polypeptide ligands; lectins; intercellular adhesion receptors such as specific leukocyte integrins, selectins, immunoglobulin gene superfamily members, intercellular adhesion molecules (ICAM-1, -2, -3) and the like; histocompatibility antigens; etc.

[0103] Examples of cell surface receptors that may provide a binding domain polypeptide, and that may also be selected as the target molecule or antigen to which a binding domain-Ig fusion protein of the present invention desirably binds, include the following, or the like: HER1 (e.g., GenBank Accession Nos. U48722, SEG_HEGFREXS, KO3193), HER2 (Yoshino et al., 1994 *J. Immunol.* 152:2393; Disis et al., 1994 *Canc. Res.* 54:16; see also, e.g., GenBank Acc. Nos. X03363, M17730, SEG_HUMHER20), HER3 (e.g., GenBank Acc. Nos. U29339, M34309), HER4 (Plowman et al., 1993 *Nature* 366:473; see also e.g., GenBank Acc. Nos. L07868, T64105), epidermal growth factor receptor (EGFR) (e.g., GenBank Acc. Nos. U48722, SEG_HEGFREXS, KO3193), vascular endothelial cell growth factor(e.g., GenBank No. M32977), vascular endothelial cell growth factor receptor (e.g., GenBank Acc. Nos. AF022375, 1680143, U48801, X62568), insulin-like growth factor-I (e.g., GenBank Acc. Nos. X00173, X56774, X56773, X06043, see also European Patent No. GB 2241703), insulin-like growth factor-II (e.g., GenBank Acc. Nos. X03562, X00910, SEG_HUMGFIA, SEG_HUMGFI2, M17863, M17862), transferrin receptor (Trowbridge and Omary, 1981 *Proc. Nat. Acad. USA* 78:3039; see also e.g., GenBank Acc. Nos. X01060, M11507), estrogen receptor (e.g., GenBank Acc. Nos. M38651, X03635, X99101, U47678, M12674), progesterone receptor (e.g., GenBank Acc. Nos. X51730, X69068, M15716), follicle stimulating hormone receptor (FSH-R) (e.g., GenBank Acc. Nos. Z34260, M65085), retinoic acid receptor (e.g., GenBank Acc. Nos. L12060, M60909, X77664, X57280, X07282, X06538), MUC-1 (Barnes et al., 1989 *Proc. Nat. Acad. Sci. USA* 86:7159; see also e.g., GenBank Acc. Nos. SEG_MUSMUCIO, M65132, M64928) NY-ESO-1 (e.g., GenBank Acc. Nos. AJ003149, U87459), NA 17-A (e.g., European Patent No. WO 96/40039), Melan-A/MART-1 (Kawakami et al., 1994 *Proc. Nat. Acad. Sci. USA* 91:3515; see also e.g., GenBank Acc. Nos. U06654, U06452), tyrosinase (Topalian et al., 1994 *Proc. Nat. Acad. Sci. USA* 91:9461; see also e.g., GenBank Acc. Nos. M26729, SEG_HUMTYR0, see also Weber et al., *J. Clin. Invest* (1998) 102:1258), Gp-100 (Kawakami et al., 1994 *Proc. Nat. Acad. Sci. USA* 91:3515; see also e.g., GenBank Acc. No. S73003, see also European Patent No. EP 668350; Adema et al., 1994 *J. Biol. Chem.* 269:20126), MAGE (van den Bruggen et al., 1991 *Science* 254:1643; see also e.g, GenBank Acc. Nos. U93163, AF064589, U66083, D32077, D32076, D32075, U10694, U10693, U10691, U10690, U10689, U10688, U10687, U10686, U10685, L18877, U10340, U10339, L18920, U03735, M77481), BAGE (e.g., GenBank Acc. No. U19180, see also U.S. Pat. Nos. 5,683,886 and 5,571,711), GAGE (e.g., GenBank Acc. Nos. AF055475, AF055474, AF055473, U19147, U19146, U19145, U19144, U19143, U19142), any of the CTA class of receptors including in particular HOM-MEL-40 antigen encoded by the SSX2 gene (e.g., GenBank Acc. Nos. X86175, U90842, U90841, X86174), carcinoembyonic antigen (CEA, Gold and Freedman, 1985 *J. Exp. Med.* 121:439; see also e.g., GenBank Acc. Nos. SEG_HUMCEA, M59710, M59255, M29540), and PyLT (e.g., GenBank Acc. Nos. J02289, J02038).

[0104] Additional cell surface receptors that may be sources of binding domain polypeptides or that may be cognate antigens include the following, or the like: CD2

(e.g., GenBank Acc. Nos. Y00023, SEG_HUMCD2, M16336, M16445, SEG_MUSCD2, M14362), 4-1BB (CDw137, Kwon et al., 1989 *Proc. Nat. Acad. Sci. USA* 86:1963, 4-1BB ligand (Goodwin et al., 1993 *Eur. J. Immunol.* 23:2361; Melero et al., 1998 *Eur. J. Immunol.* 3:116), CD5 (e.g., GenBank Acc. Nos. X78985, X89405), CD10 (e.g., GenBank Acc. Nos. M81591, X76732) CD27 (e.g., GenBank Acc. Nos. M63928, L24495, L08096), CD28 (June et al., 1990 *Immunol. Today* 11:211; see also, e.g., GenBank Acc. Nos. J02988, SEG_HUMCD28, M34563), CD152/CTLA-4 (e.g., GenBank Acc. Nos. L15006, X05719, SEG_HUMIGCTL), CD40 (e.g., GenBank Acc. Nos. M83312, SEG_MUSC040A0, Y10507, X67878, X96710, U15637, L07414), interferon-γ (IFN-γ; see, e.g., Farrar et al. 1993 *Ann. Rev. Immunol.* 11:571 and references cited therein, Gray et al. 1982 *Nature* 295:503, Rinderknecht et al. 1984 *J. Biol. Chem.* 259:6790, DeGrado et al. 1982 *Nature* 300:379), interleukin-4 (IL-4; see, e.g., 53rd *Forum in Immunology,* 1993 *Research in Immunol.* 144:553-643; Banchereau et al., 1994 in *The Cytokine Handbook,* 2nd ed., A. Thomson, ed., Academic Press, NY, p. 99; Keegan et al., 1994 *J Leukocyt. Biol.* 55:272, and references cited therein), interleukin-17 (IL-17) (e.g., GenBank Acc. Nos. U32659, U43088) and interleukin-17 receptor (IL-17R) (e.g., GenBank Acc. Nos. U31993, U58917). Notwithstanding the foregoing, the present invention expressly does not encompass any immunoglobulin fusion protein that is disclosed in U.S. Pat. No. 5,807,734, U.S. Pat. No. 5,795,572 or U.S. Pat. No. 5,807,734.

[0105] Additional cell surface receptors that may be sources of binding domain polypeptides or that may be cognate antigens include the following, or the like: CD59 (e.g., GenBank Acc. Nos. SEG_HUMCD590, M95708, M34671), CD48 (e.g., GenBank Acc. Nos. M59904), CD58/LFA-3 (e.g., GenBank Acc. No. A25933, Y00636, E12817; see also JP 1997075090-A), CD72 (e.g., GenBank Acc. Nos. AA311036, S40777, L35772), CD70 (e.g., GenBank Acc. Nos. Y13636, S69339), CD80/B7.1 (Freeman et al., 1989 *J. Immunol.* 43:2714; Freeman et al., 1991 *J. Exp. Med.* 174:625; see also e.g., GenBank Acc. Nos. U33208, I683379), CD86/B7.2 (Freeman et al., 1993 *J. Exp. Med.* 178:2185, Boriello et al., 1995 *J. Immunol.* 155:5490; see also, e.g., GenBank Acc. Nos. AF099105, SEG_MMB72G, U39466, U04343, SEG_HSB725, L25606, L25259), B7-H1/B7-DC (e.g., Genbank Acc. Nos. NM_014143, AF177937, AF317088; Dong et al., 2002 *Nat. Med.* June 24 [epub ahead of print], PMID 12091876; Tseng et al., 2001 *J. Exp. Med.* 193:839; Tamura et al., 2001 *Blood* 97:1809; Dong et al., 1999 *Nat. Med.* 5:1365), CD40 ligand (e.g., GenBank Acc. Nos. SEG_HUMCD40L, X67878, X65453, L07414), IL-17 (e.g., GenBank Acc. Nos. U32659, U43088), CD43 (e.g., GenBank Acc. Nos. X52075, J04536), ICOS (e.g., Genbank Acc. No. AH011568), CD3 (e.g., Genbank Acc. Nos. NM_000073 (gamma subunit), NM_000733 (epsilon subunit), X73617 (delta subunit)), CD4 (e.g., Genbank Acc. No. NM_000616), CD25 (e.g., Genbank Acc. No. NM_000417), CD8 (e.g., Genbank Acc. No.M12828), CD11b (e.g., Genbank Acc. No. J03925), CD14 (e.g., Genbank Acc. No. XM_039364), CD56 (e.g., Genbank Acc. No.U63041), CD69 (e.g., Genbank Acc. No.NM_001781) and VLA-4 ($\alpha_4\beta_7$) (e.g., GenBank Acc. Nos. L12002, X16983, L20788, U97031, L24913, M68892, M95632). The following cell surface receptors are typically associated with B cells: CD19 (e.g., GenBank Acc. Nos. SEG_HUMCD19W0, M84371, SEG_MUSCD19W, M62542), CD20 (e.g., GenBank Acc. Nos. SEG-_HUMCD20, M62541), CD22 (e.g., GenBank Acc. Nos. I680629, Y10210, X59350, U62631, X52782, L16928), CD30 (e.g., Genbank Acc. Nos. M83554, D86042), CD153 (CD30 ligand, e.g., GenBank Acc. Nos. L09753, M83554), CD37 (e.g., GenBank Acc. Nos. SEG_MMCD37X, X14046, X53517), CD50 (ICAM-3, e.g., GenBank Acc. No. NM_002162), CD106 (VCAM-1) (e.g., GenBank Acc. Nos. X53051, X67783, SEG_MMVCAM1C, see also U.S. Pat. No. 5,596,090), CD54 (ICAM-1) (e.g., GenBank Acc. Nos. X84737, S82847, X06990, J03132, SEG_MUSICAM0), interleukin-12 (see, e.g., Reiter et al, 1993 *Crit. Rev. Immunol.* 13:1, and references cited therein), CD134 (OX40, e.g., GenBank Acc. No. AJ277151), CD137 (41BB, e.g., GenBank Acc. No. L12964, NM_001561), CD83 (e.g., GenBank Acc. Nos. AF001036, AL021918), DEC-205 (e.g., GenBank Acc. Nos. AF011333, U19271).

[0106] Binding domain-immunoglobulin fusion proteins of the present invention comprise a binding domain polypeptide that, according to certain particularly preferred embodiments, is capable of specifically binding at least one antigen that is present on an immune effector cell. According to non-limiting theory, such binding domain-Ig fusion proteins may advantageously recruit desired immune effector cell function(s) in a therapeutic context, where it is well known that immune effector cells having different specialized immune functions can be distinguished from one another on the basis of their differential expression of a wide variety of cell surface antigens, such as many of the antigens described herein to which binding domain polypeptides can specifically bind. Immune effector cells include any cell that is capable of directly mediating an activity which is a component of immune system function, including cells having such capability naturally or as a result of genetic engineering.

[0107] In certain embodiments an immune effector cell comprises a cell surface receptor for an immunoglobulin, such as a receptor for an immunoglobulin constant region and including the class of receptors commonly referred to as "Fc receptors" (FcR). A number of FcR have been structurally and/or functionally characterized and are well known in the art, including FcR having specific abilities to interact with a restricted subset of immunoglobulin heavy chain isotypes, or that interact with Fc domains with varying affinities, and/or which may be expressed on restricted subsets of immune effector cells under certain conditions (e.g., Kijimoto-Ochichai et al., 2002 *Cell Mol. Life Sci.* 59:648; Davis et al., 2002 *Curr. Top. Microbiol. Immunol.* 266:85; Pawankar, 2001 *Curr. Opin. Allerg. Clin. Immunol.* 1:3; Radaev et al., 2002 *Mol. Immunol.* 38:1073; Wurzburg et al., 2002 *Mol. Immunol.* 38:1063; Sulica et al., 2001 *Int. Rev. Immunol.* 20:371; Underhill et al., 2002 *Ann. Rev. Immunol.* 20:825; Coggeshall, 2002 *Curr. Dir. Autoimm.* 5:1; Mimura et al., 2001 *Adv. Exp. Med. Biol.* 495:49; Baumann et al., 2001 *Adv. Exp. Med. Biol.* 495:219; Santoso et al., 2001 *Ital. Heart J.* 2:811; Novak et al., 2001 *Curr. Opin. Immunol.* 13:721; Fossati et al., 2001 *Eur. J. Clin. Invest.* 31:821).

[0108] Cells that are capable of mediating ADCC are preferred examples of immune effector cells according to the present invention. Other preferred examples include natural killer (NK) cells, tumor-infiltrating T lymphocytes (TIL),

cytotoxic T lymphocytes (CTL), and granulocytic cells such as cells that comprise allergic response mechanisms. Immune effector cells thus include, but are not limited to, cells of hematopoietic origins including cells at various stages of differentiation within myeloid and lymphoid lineages and which may (but need not) express one or more types of functional cell surface FcR, such as T lymphocytes, B lymphocytes, NK cells, monocytes, macrophages, dendritic cells, neutrophils, basophils, eosinophils, mast cells, platelets, erythrocytes, and precursors, progenitors (e.g., hematopoietic stem cells), quiescent, activated and mature forms of such cells. Other immune effector cells may include cells of non-hematopoietic origin that are capable of mediating immune functions, for example, endothelial cells, keratinocytes, fibroblasts, osteoclasts, epithelial cells and other cells. Immune effector cells may also include cells that mediate cytotoxic or cytostatic events, or endocytic, phagocytic, or pinocytotic events, or that effect induction of apoptosis, or that effect microbial immunity or neutralization of microbial infection, or cells that mediate allergic, inflammatory, hypersensitivity and/or autoimmune reactions.

[0109] Allergic response mechanisms are well known in the art and include an antigen (e.g., allergen)-specific component such as an immunoglobulin (e.g., IgE), as well as the cells and mediators which comprise sequelae to allergen-immunoglobulin (e.g., IgE) encounters (e.g., Ott et al., 2000 *J. Allerg. Clin. Immunol.* 106:429; Barnes, 2000 *J. Allerg. Clin. Immunol.* 106:5; Togias, 2000 *J. Allerg. Clin. Immunol.* 105:S599; Akdis et al., 2000 *Int. Arch. Allerg. Immunol.* 121:261; Beach, 2000 *Occup. Med.* 15:455). Particularly with regard to binding domain-immunoglobulin fusion proteins of the present invention that interact with FcR, certain embodiments of the present invention contemplate fusion proteins that comprise one or more IgE-derived domains and that are capable of inducing an allergic response mechanism that comprises IgE-specific FcR, as also noted above and as described in the cited references. Without wishing to be bound by theory, and as disclosed herein, fusion proteins of the present invention may comprise portions of IgE heavy chain Fc domain polypeptides, whether expressed as cell surface proteins (e.g., with a plasma membrane anchor domain) or as soluble proteins (e.g., without a plasma membrane anchor domain). Further according to non-limiting theory, recruitment and induction of an allergic response mechanism (e.g., an FcR-epsilon expressing immune effector cell) may proceed as the result of either or both of the presence of an IgE Fc domain (e.g., that is capable of triggering an allergic mechanism by FcR crosslinking) and the presence of the cognate antigen to which the binding domain specifically binds. The present invention therefore exploits induction of allergic response mechanisms in heretofore unappreciated contexts, such as treatment of a malignant condition or a B-cell disorder as described herein.

[0110] An immunoglobulin hinge region polypeptide, as discussed above, includes any hinge peptide or polypeptide that occurs naturally, as an artificial peptide or as the result of genetic engineering and that is situated in an immunoglobulin heavy chain polypeptide between the amino acid residues responsible for forming intrachain immunoglobulin-domain disulfide bonds in CH1 and CH2 regions; hinge region polypeptides for use in the present invention may also include a mutated hinge region polypeptide. Accordingly, an immunoglobulin hinge region polypeptide may be derived from, or may be a portion or fragment of (i.e., one or more amino acids in peptide linkage, typically 15-115 amino acids, preferably 95-110, 80-94, 60-80, or 5-65 amino acids, preferably 10-50, more preferably 15-35, still more preferably 18-32, still more preferably 20-30, still more preferably 21, 22, 23, 24, 25, 26, 27, 28 or 29 amino acids) an immunoglobulin polypeptide chain region classically regarded as having hinge function, as described above, but a hinge region polypeptide for use in the instant invention need not be so restricted and may include amino acids situated (according to structural criteria for assigning a particular residue to a particular domain that may vary, as known in the art) in an adjoining immunoglobulin domain such as a CH1 domain or a CH2 domain, or in the case of certain artificially engineered immunoglobulin constructs, an immunoglobulin variable region domain.

[0111] Wild-type immunoglobulin hinge region polypeptides include any naturally occurring hinge region that is located between the constant region domains, CH1 and CH2, of an immunoglobulin. The wild-type immunoglobulin hinge region polypeptide is preferably a human immunoglobulin hinge region polypeptide, preferably comprising a hinge region from a human IgG, IgA or IgE immunoglobulin, and more preferably, a hinge region polypeptide from a wild-type or mutated human IgG1 isotype as described herein. As is known to the art, despite the tremendous overall diversity in immunoglobulin amino acid sequences, immunoglobulin primary structure exhibits a high degree of sequence conservation in particular portions of immunoglobulin polypeptide chains, notably with regard to the occurrence of cysteine residues which, by virtue of their sulfyhydryl groups, offer the potential for disulfide bond formation with other available sulfydryl groups. Accordingly, in the context of the present invention wild-type immunoglobulin hinge region polypeptides may be regarded as those that feature one or more highly conserved (e.g., prevalent in a population in a statistically significant manner) cysteine residues, and in certain preferred embodiments a mutated hinge region polypeptide may be selected that contains zero or one cysteine residue and that is derived from such a wild-type hinge region.

[0112] In certain preferred embodiments wherein the hinge region polypeptide is a mutated human IgG1 immunoglobulin hinge region polypeptide that is derived from a wild-type hinge region, it is noted that the wild-type human IgG1 hinge region polypeptide sequence comprises three non-adjacent cysteine residues, referred to as a first cysteine of the wild-type hinge region, a second cysteine of the wild-type hinge region and a third cysteine of the wild-type hinge region, respectively, proceeding along the hinge region sequence from the polypeptide N-terminus toward the C-terminus. Accordingly, in certain such embodiments of the present invention, the mutated human IgG1 immunoglobulin hinge region polypeptide contains two cysteine residues and the first cysteine of the wild-type hinge region is not mutated. In certain other embodiments of the present invention the mutated human IgG1 immunoglobulin hinge region polypeptide contains no more than one cysteine residue, and in certain other embodiments the mutated human IgG1 immunoglobulin hinge region polypeptide contains no cysteine residues.

[0113] The binding domain-immunoglobulin fusion proteins of the present invention expressly do not contemplate

any fusion protein that is disclosed in U.S. Pat. No. 5,892, 019. For example, and as disclosed in U.S. Pat. No. 5,892, 019, a mutated human IgG1 hinge region described therein has a substitution or deletion of the first IgG1 hinge region cysteine residue, but retains both of the second and third IgG1 hinge region cysteine residues that correspond to the second and third cysteines of the wild-type IgG1 hinge region sequence. This reference discloses that the first cysteine residue of the wild-type IgG1 hinge region is replaced to prevent interference, by the first cysteine residue, with proper assembly of the single chain immunoglobulin-like polypeptide described therein into an immunoglobulin-like dimer. As also disclosed in this reference, the second and third cysteines of the IgG1 hinge region are retained to provide interchain disulfide linkage between two heavy chain constant regions to promote dimer formation, which further according to U.S. Pat. No. 5,892,019 results in an immunoglobulin-like dimer having effector function such as ADCC capability.

[0114] By contrast and as described herein, the binding domain-immunogloblin fusion proteins of the present invention, which are capable of ADCC, are not so limited and may comprise, in pertinent part, (i) a wild-type human IgG1 immunoglobulin hinge region polypeptide, (ii) a mutated human IgG1 immunoglobulin hinge region polypeptide that is derived from a wild-type immunoglobulin hinge region polypeptide having three or more cysteine residues, wherein the mutated human IgG1 immunoglobulin hinge region polypeptide contains two cysteine residues and wherein a first cysteine of the wild-type hinge region is not mutated, (iii) a mutated human IgG1 immunoglobulin hinge region polypeptide that is derived from a wild-type immunoglobulin hinge region polypeptide having three or more cysteine residues, wherein the mutated human IgG1 immunoglobulin hinge region polypeptide contains no more than one cysteine residue, or (iv) a mutated human IgG1 immunoglobulin hinge region polypeptide that is derived from a wild-type immunoglobulin hinge region polypeptide having three or more cysteine residues, wherein the mutated human IgG1 immunoglobulin hinge region polypeptide contains no cysteine residues. In particular, the present invention thus offers unexpected advantages associated with retention by the fusion proteins described herein of the ability to mediate ADCC even where the ability to dimerize via IgG1 hinge region interchain disulfide bonds is ablated or compromised by the removal or replacement of one, two or three hinge region cysteine residues, and even where the first cysteine of the IgG1 hinge region is not mutated.

[0115] A mutated immunoglobulin hinge region polypeptide may comprise a hinge region that has its origin in an immunoglobulin of a species, of an immunoglobulin isotype or class, or of an immunoglobulin subclass that is different from that of the CH2 and CH3 domains. For instance, in certain embodiments of the invention, the binding domain-immunoglobulin fusion protein may comprise a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide comprising a wild-type human IgA hinge region polypeptide, or a mutated human IgA hinge region polypeptide that contains zero or only one cysteine residues, as described herein, or a wild-type human IgG1 hinge region polypeptide or a wild-type human IgE hinge region polypeptide or a mutated human IgG1 hinge region polypeptide that is mutated to contain zero, one or two cysteine residues wherein the first cysteine of the wild-type hinge region is not mutated, as also described herein. Such a hinge region polypeptide may be fused to an immunoglobulin heavy chain CH2 region polypeptide from a different Ig isotype or class, for example an IgA or an IgE or an IgG subclass, which in certain preferred embodiments will be the IgG1 subclass and in certain other preferred embodiments may be any one of the IgG2, IgG3 or IgG4 subclasses.

[0116] For example, and as described in greater detail below, in certain embodiments of the present invention an immunoglobulin hinge region polypeptide is selected which is derived from a wild-type human IgA hinge region that naturally comprises three cysteines, where the selected hinge region polypeptide is truncated relative to the complete hinge region such that only one of the cysteine residues remains (e.g., SEQ ID NOS:35-36). Similarly, in certain other embodiments of the invention, the binding domain-immunoglobulin fusion protein comprises a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide comprising a mutated hinge region polypeptide in which the number of cysteine residues is reduced by amino acid substitution or deletion, for example a mutated IgG1 hinge region containing zero, one or two cysteine residues as described herein. A mutated hinge region polypeptide may thus be derived from a wild-type immunoglobulin hinge region that contains one or more cysteine residues. In certain embodiments, a mutated hinge region polypeptide may contain zero or only one cysteine residue, wherein the mutated hinge region polypeptide is derived from a wild type immunoglobulin hinge region that contains, respectively, one or more or two or more cysteine residues. In the mutated hinge region polypeptide, the cysteine residues of the wild-type immunoglobulin hinge region are preferably substituted with amino acids that are incapable of forming a disulfide bond. In one embodiment of the invention, the mutated hinge region polypeptide is derived from a human IgG wild-type hinge region polypeptide, which may include any of the four human IgG isotype subclasses, IgG1, IgG2, IgG3 or IgG4. In certain preferred embodiments, the mutated hinge region polypeptide is derived from a human IgG1 wild-type hinge region polypeptide. By way of example, a mutated hinge region polypeptide derived from a human IgG1 wild-type hinge region polypeptide may comprise mutations at two of the three cysteine residues in the wild-type immunoglobulin hinge region, or mutations at all three cysteine residues.

[0117] The cysteine residues that are present in a wild-type immunoglobulin hinge region and that are removed by mutagenesis according to particularly preferred embodiments of the present invention include cysteine residues that form, or that are capable of forming, interchain disulfide bonds. Without wishing to be bound by theory, the present invention contemplates that mutation of such hinge region cysteine residues, which are believed to be involved in formation of interchain disulfide bridges, reduces the ability of the subject invention binding domain-immunoglobulin fusion protein to dimerize (or form higher oligomers) via interchain disulfide bond formation, while surprisingly not ablating the ability of the fusion protein to promote antibody dependent cell-mediated cytotoxicity (ADCC) or to fix complement. In particular, the Fc receptors (FcR) which mediate ADCC (e.g., FCRIII, CD16) exhibit low affinity for immunoglobulin Fc domains, suggesting that functional binding of Fc to FcR requires avidity stabilization of the Fc-FcR complex by virtue of the dimeric structure of heavy

chains in a conventional antibody, and/or FcR aggregation and cross-linking by a conventional Ab Fc structure. (Sonderman et al., 2000 *Nature* 406:267; Radaev et al., 2001 *J. Biol. Chem.* 276:16469; Radaev et al., 2001 *J. Biol. Chem.* 276:16478; Koolwijk et al., 1989 *J. Immunol* 143:1656; Kato et al., 2000 *Immunol. Today* 21:310.) Hence, the binding domain-immunoglobulin fusion proteins of the present invention provide the advantages associated with single-chain immunoglobulin fusion proteins while also unexpectedly retaining immunological activity. Similarly, the ability to fix complement is typically associated with immunoglobulins that are dimeric with respect to heavy chain constant regions such as those that comprise Fc, while the binding domain-immunoglobulin fusion proteins of the present invention, which may, due to the replacement or deletion of hinge region cysteine residues or due to other structural modifications as described herein, have compromised or ablated abilities to form interchain disulfide bonds, exhibit the unexpected ability to fix complement. Additionally, according to certain embodiments of the present invention wherein a binding domain-immunoglobulin fusion protein may comprise one or more of a human IgE hinge region polypeptide, a human IgE CH2 constant region polypeptide, a human IgE CH3 constant region polypeptide, and a human IgE CH4 constant region polypeptide, the invention fusion proteins unexpectedly retain the immunological activity of mediating ADCC and/or of inducing an allergic response mechanism.

[0118] Selection of an immunoglobulin hinge region polypeptide according to certain embodiments of the subject invention binding domain-immunoglobulin fusion proteins may relate to the use of an "alternative hinge region" polypeptide sequence, which includes a polypeptide sequence that is not necessarily derived from any immunoglobulin hinge region sequence per se. Instead, an alternative hinge region refers to a hinge region polypeptide that comprises an amino acid sequence of at least ten consecutive amino acids, and in certain embodiments at least 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21-25, 26-30, 31-50, 51-60, 71-80, 81-90, or 91-110 amino acids that is present in a sequence selected from any one of SEQ ID NOS:___-___, for example a polypeptide sequence derived from a region located between intrachain disulfide-generated immunoglobulin-like loop domains of immunoglobulin gene superfamily members such as CD2 (e.g., Genbank Acc. No. NM_001767), CD4 (e.g., Genbank Acc. No. NM_000616), CD5 (e.g., Genbank Acc. No. BC027901), CD6 (e.g., Genbank Acc. No. NM_006725), CD7 (e.g., Genbank Acc. Nos. XM_046782, BC009293, NM_006137) or CD8 (e.g., Genbank Acc. No. M12828), or other Ig superfamily members. By way of non-limiting example, an alternative hinge region may provide a glycosylation site as provided herein, or may provide a human gene-derived polypeptide sequence for purposes of enhancing the degree of "humanization" of a fusion protein, or may comprise an amino acid sequence that reduces the ability of a fusion protein to form multimers or oligomers or aggregates or the like. Certain alternative hinge region polypeptide sequences as described herein may be derived from the polypeptide sequences of immunoglobulin gene superfamily members that are not actual immunoglobulins per se. For instance and according to non-limiting theory, certain polypeptide sequences that are situated between intrachain disulfide-generated immunoglobulin loop domain of immunoglobulin gene super-family member proteins may be used as alternative hinge region polypeptides as provided herein, or may be further modified for such use.

[0119] As noted above, binding domain-immunoglobulin fusion proteins are believed, according to non-limiting theory, to be compromised in their ability to dimerize via interchain disulfide bond formation, and further according to theory, this property is a consequence of a reduction in the number of cysteine residues that are present in the immunoglobulin hinge region polypeptide selected for inclusion in the construction of the fusion protein. Determination of the relative ability of a polypeptide to dimerize is well within the knowledge of the relevant art, where any of a number of established methodologies may be applied to detect protein dimerization (see, e.g., Scopes, *Protein Purification: Principles and Practice,* 1987 Springer-Verlag, New York). For example, biochemical separation techniques for resolving proteins on the basis of molecular size (e.g., gel electrophoresis, gel filtration chromatography, analytical ultracentrifugation, etc.), and/or comparison of protein physicochemical properties before and after introduction of sulfhydryl-active (e.g., iodoacetamide, N-ethylmaleimide) or disulfide-reducing (e.g., 2-mercaptoethanol, dithiothreitol) agents, or other equivalent methodologies, may all be employed for determining a degree of polypeptide dimerization or oligomerization, and for determining possible contribution of disulfide bonds to such potential quarternary structure. In certain embodiments, the invention relates to a binding domain-immunoglobulin fusion protein that exhibits a reduced (i.e., in a statistically significant manner relative to an appropriate IgG-derived control) ability to dimerize, relative to a wild-type human immunoglobulin G hinge region polypeptide as provided herein. Accordingly, those familiar with the art will be able readily to determine whether a particular fusion protein displays such reduced ability to dimerize.

[0120] Compositions and methods for preparation of immunoglobulin fusion proteins are well known in the art, as described for example, in U.S. Pat. No. 5,892,019, which discloses recombinant antibodies that are the products of a single encoding polynucleotide but which are not binding domain-immunoglobulin fusion proteins according to the present invention.

[0121] For an immunoglobulin fusion protein of the invention which is intended for use in humans, the constant regions will typically be of human sequence origin, to minimize a potential anti-human immune response and to provide appropriate effector functions. Manipulation of sequences encoding antibody constant regions is described in the PCT publication of Morrison and Oi, WO 89/07142. In particularly preferred embodiments, the CH1 domain is deleted and the carboxyl end of the binding domain, or where the binding domain comprises two immunoglobulin variable region polypeptides, the second (i.e., more proximal to the C-terminus) variable region is joined to the amino terminus of CH2 through the hinge region. A schematic diagram depicting the structures of two exemplary binding domain-immunoglobulin fusion proteins is shown in **FIG. 11**, where it should be noted that in particularly preferred embodiments no interchain disulfide bonds are present, and in other embodiments a restricted number of interchain disulfide bonds may be present relative to the number of such bonds that would be present if wild-type hinge region

polypeptides were instead present, and that in other embodiments the fusion protein comprises a mutated hinge region polypeptide that exhibits a reduced ability to dimerize, relative to a wild-type human IgG hinge region polypeptide. Thus, the isolated polynucleotide molecule codes for a single chain immunoglobulin fusion protein having a binding domain that provides specific binding affinity for a selected antigen.

[0122] The invention also contemplates in certain embodiments binding domain-immunoglobulin fusion proteins as provided herein that comprise fused polypeptide sequences or portions thereof derived from a plurality of genetic sources, for example, according to molecular "domain swapping" paradigms. Those having familiarity with the art will readily appreciate that selection of such polypeptide sequences for assembly into a binding domain-immunoglobulin fusion protein may involve determination of what are appropriate portions of each component polypeptide sequence, based on structural and/or functional properties of each such sequence (see, e.g., Carayannopoulos et al., 1996 *J. Exp. Med.* 183:1579; Harlow et al., Eds., *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor (1988)). The component polypeptide sequences of which the fusion protein is comprised may therefore comprise intact or full-length binding domain, immunoglobulin, linker and/or plasma membrane anchor domain polypeptide sequences, or truncated versions or variants thereof as provided herein. According to these and related embodiments of the invention, any two or more of the candidate component polypeptides of which the subject invention fusion protein may be comprised will be derived from independent sources, such as from immunoglobulin sequences of differing allotype, isotype, subclass, class, or species of origin (e.g., xenotype). Thus, as a non-limiting example, a binding domain polypeptide (or its constituent polypeptides such as one or more variable region polypeptides and/or a linker polypeptide), a hinge region polypeptide, immunoglobulin heavy chain CH2 and CH3 constant region polypeptides and optionally an immunoglobulin heavy chain CH4 constant region polypeptide as may be obtained from an IgM or IgE heavy chain, and a plasma membrane anchor domain polypeptide may all be separately obtained from distinct genetic sources and engineered into a chimeric or fusion protein using well known techniques and according to methodologies described herein.

[0123] Accordingly, a binding domain-immunoglobulin fusion protein according to certain embodiments of the present invention may also therefore comprise in pertinent part an immunoglobulin heavy chain CH3 constant region polypeptide that is a wild-type IgA CH3 constant region polypeptide, or alternatively, that is a mutated IgA CH3 constant region polypeptide that is incapable of associating with a J chain; preferably the IgA CH3 constant region polypeptides are of human origin. By way of brief background, IgA molecules are known to be released into secretory fluids by a mechanism that involves association of IgA into disulfide-linked polymers (e.g., dimers) via a J chain polypeptide (e.g., GenBank Acc. Nos. XM_059628, M12378, M12759; Johansen et al., 1999 *Eur. J. Immunol.* 29:1701) and interaction of the complex so formed with another protein that acts as a receptor for polymeric immunoglobulin, and which is known as transmembrane secretory component (S C; Johansen et al., 2000 *Sc. J. Immunol.* 52:240; see also, e.g., Sorensen et al., 2000 *Int. Immunol.* 12:19; Yoo et al., 1999 *J. Biol. Chem.* 274:33771; Yoo et al., 2002 *J. Immunol. Meth.* 261:1; Corthesy, 2002 *Trends Biotechnol.* 20:65; Symersky et al., 2000 *Mol. Immunol.* 37:133; Crottet et al., 1999 *Biochem.* J. 341:299). Interchain disulfide bond formation between IgA Fc domains and J chain is mediated through a penultimate cysteine residue in an 18-amino acid C-terminal extension that forms part of the IgA heavy chain constant region CH3 domain polypeptide (Yoo et al., 1999; Sorensen et al., 2000). Certain embodiments of the subject invention fusion proteins therefore contemplate inclusion of the wild-type IgA heavy chain constant region polypeptide sequence, which is capable of associating with J chain. Certain other embodiments of the invention, however, contemplate fusion proteins that comprise a mutated IgA CH3 constant region polypeptide that is incapable of associating with a J chain. According to such embodiments, two or more residues from the C-terminus of an IgA CH3 constant region polypeptide such as a human IgA CH3 constant region polypeptide may be deleted to yield a truncated CH3 constant region polypeptide as provided herein. In preferred embodiments and as described in greater detail below, a mutated human IgA CH3 constant region polypeptide that is incapable of associating with a J chain comprises such a C-terminal deletion of either four or 18 amino acids. However, the invention need not be so limited, such that the mutated IgA CH3 constant region polypeptide may comprise a deletion of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21-25, 26-30 or more amino acids, so long as the fusion protein is capable of specifically binding an antigen and of at least one immunological activity as provided herein. Alternatively, the invention also contemplates fusion proteins that comprise a mutated IgA CH3 constant region polypeptide that is incapable of associating with a J chain by virtue of replacement of the penultimate cysteine, or by chemical modification of that amino acid residue, in a manner that prevents interchain disulfide bond formation. Methods for determining whether a fusion protein can associate with a J chain will be known to those having familiarity with the art and are described herein, including in the cited references.

[0124] As also described herein and according to procedures known in the art, the fusion protein may further be tested routinely for immunological activity, for instance, in ADCC or CDC assays. As an illustrative example, a fusion protein according to such an embodiment may comprise a binding domain polypeptide derived from a human heavy chain variable region polypeptide sequence, a human IgA-derived immunoglobulin hinge region polypeptide sequence, a human IgG1 immunoglobulin heavy chain CH2 constant region polypeptide sequence, a human IgG2 immunoglobulin heavy chain CH3 constant region polypeptide sequence, and optionally a human IgE immunoglobulin heavy chain CH4 constant region polypeptide sequence and/or a human TNF-α receptor type 1 (TNFR1) plasma membrane anchor domain polypeptide sequence that comprises a cytoplasmic tail polypeptide which is capable of apoptotic signaling. The invention therefore contemplates these and other embodiments according to the present invention in which two or more polypeptide sequences that are present in a fusion protein have independent genetic origins.

[0125] As noted above, in certain embodiments the binding protein-immunoglobulin fusion protein comprises at least one immunoglobulin variable region polypeptide, which may be a light chain or a heavy chain variable region

polypeptide, and in certain embodiments the fusion protein comprises at least one such light chain V-region and one such heavy chain V-region and at least one linker peptide that is fused to each of the V-regions. Construction of such binding domains, for example single chain Fv domains, is well known in the art and is described in greater detail in the Examples below, and has been described, for example, in U.S. Pat. No. 5,892,019 and references cited therein; selection and assembly of single-chain variable regions and of linker polypeptides that may be fused to each of a heavy chain-derived and a light chain-derived V region (e.g., to generate a binding domain that comprises a single-chain Fv polypeptide) is also known to the art and described herein and, for example, in U.S. Pat. No. 5,869,620, U.S. Pat. No. 4,704,692 and U.S. Pat. No. 4,946,778. In certain embodiments all or a portion of an immunoglobulin sequence that is derived from a non-human source may be "humanized" according to recognized procedures for generating humanized antibodies, i.e., immunoglobulin sequences into which human Ig sequences are introduced to reduce the degree to which a human immune system would perceive such proteins as foreign (see, e.g., U.S. Pat. Nos. 5,693,762; 5,585, 089; 4,816,567; 5,225,539; 5,530,101; and references cited therein).

[0126] Binding domain-immunoglobulin fusion proteins as described herein may, according to certain embodiments, desirably comprise sites for glycosylation, e.g., covalent attachment of carbohydrate moieties such as monosaccharides or oligosaccharides. Incorporation of amino acid sequences that provide substrates for polypeptide glycosylation is within the scope of the relevant art, including, for example, the use of genetic engineering or protein engineering methodologies to obtain a polypeptide sequence containing the classic Asn-X-Ser/Thr site for N-(asparagine)-linked glycosylation, or a sequence containing Ser or Thr residues that are suitable substrates for O-linked glycosylation, or sequences amenable to C-mannosylation, glypiation/glycosylphosphatidylinositol modification, or phosphoglycation, all of which can be identified according to art-established criteria (e.g., Spiro, 2002 *Glybiology* 12:43R). Without wishing to be bound by theory, glycosylated fusion proteins having particular amino acid sequences may beneficially possess attributes associated with one or more of improved solubility, enhanced stability in solution, enhanced physiological stability, improved bioavailability including in vivo biodistribution, and superior resistance to proteases, all in a statistically significant manner, relative to fusion proteins having the same or highly similar amino acid sequences but lacking glycosyl moieties. In certain preferred embodiments the subject invention fusion protein comprises a glycosylation site that is present in a linker as provided herein, and in certain other preferred embodiments the subject invention fusion protein comprises a glycosylation site that is present in a hinge region polypeptide sequence as provided herein.

[0127] In certain preferred embodiments of the present invention, the binding domain-immunoglobulin fusion protein is a protein or glycoprotein that is capable of being expressed by a host cell such that it localizes to the cell surface. Binding domain-immunoglobulin fusion proteins that localize to the cell surface may do so by virtue of having naturally present or artificially introduced structural features that direct the fusion protein to the cell surface (e.g., Nelson et al. 2001 *Trends Cell Biol.* 11:483; Ammon et al., 2002 *Arch. Physiol. Biochem.* 110:137; Kasai et al., 2001 *J. Cell Sci.* 114:3115; Watson et al., 2001 *Am. J. Physiol. Cell Physiol.* 281:C215; Chatterjee et al., 200 *J. Biol. Chem.* 275:24013) including by way of illustration and not limitation, secretory signal sequences, leader sequences, plasma membrane anchor domain polypeptides such as hydrophobic transmembrane domains (e.g., Heuck et al., 2002 *Cell Biochem. Biophys.* 36:89; Sadlish et al., 2002 *Biochem J.* 364:777; Phoenix et al., 2002 *Mol. Membr. Biol.* 19:1; Minke et al., 2002 *Physiol. Rev.* 82:429) or glycosylphosphatidylinositol attachment sites ("glypiation" sites, e.g., Chatterjee et al., 2001 *Cell Mol. Life Sci.* 58:1969; Hooper, 2001 *Proteomics* 1:748; Spiro, 2002 *Glycobiol.* 12:43R), cell surface receptor binding domains, extracellular matrix binding domains, or any other structural feature that causes the fusion protein to localize to the cell surface. Particularly preferred are fusion proteins that comprise a plasma membrane anchor domain which includes a transmembrane polypeptide domain, typically comprising a membrane spanning domain which includes a hydrophobic region capable of energetically favorable interaction with the phospholipid fatty acyl tails that form the interior of the plasma membrane bilayer. Such features are well known to those of ordinary skill in the art, who will further be familiar with methods for introducing nucleic acid sequences encoding these features into the subject expression constructs by genetic engineering, and with routine testing of such constructs to verify cell surface localization of the product.

[0128] According to certain further embodiments, a plasma membrane anchor domain polypeptide comprises such a transmembrane domain polypeptide and also comprises a cytoplasmic tail polypeptide, which refers to a region or portion of the polypeptide sequence that contacts the cytoplasmic face of the plasma membrane and/or is in contact with the cytosol or other cytoplasmic components. A large number of cytoplasmic tail polypeptides are known that comprise the intracellular portions of plasma membrane transmembrane proteins, and discrete functions have been identified for many such polypeptides, including biological signal transduction (e.g., activation or inhibition of protein kinases, protein phosphatases, G-proteins, cyclic nucleotides and other second messengers, ion channels, secretory pathways), biologically active mediator release, stable or dynamic association with one or more cytoskeletal components, cellular differentiation, cellular activation, mitogenesis, cytostasis, apoptosis and the like (e.g., Maher et al., 2002 *Immunol. Cell Biol.* 80:131; El Far et al., 2002 *Biochem J.* 365:329; Teng et al., 2002 *Genome Biol.* 2REVIEWS:3012; Simons et al., 2001 *Cell Signal* 13:855; Furie et al., 2001 *Thromb. Haemost.* 86:214; Gaffen, 2001 *Cytokine* 14:63; Dittel, 2000 *Arch. Immunol. Ther. Exp.* (Warsz.) 48:381; Parnes et al., 2000 *Immunol. Rev.* 176:75; Moretta et al., 2000 *Semin. Immunol.* 12:129; Ben Ze'ev, 1999 *Ann. N.Y. Acad. Sci.* 886:37; Marsters et al., *Recent Prog. Horm. Res.* 54:225).

[0129] In the context of methods of using binding domain-immunoglobulin fusion proteins for the treatment of a malignant condition or a B-cell disorder as provided herein, the present invention contemplates certain embodiments wherein a binding domain-immunoglobulin fusion protein that comprises a plasma membrane anchor domain polypeptide is expressed at a cell surface and further comprises a cytoplasmic tail polypeptide which comprises an apoptosis signaling polypeptide sequence. A number of apoptosis

signaling polypeptide sequences are known to the art, as reviewed, for example, in *When Cells Die: A Comprehensive Evaluation of Apoptosis and Programmed Cell Death* (R. A. Lockshin et al., Eds., 1998 John Wiley & Sons, New York; see also, e.g., Green et al., 1998 *Science* 281:1309 and references cited therein; Ferreira et al., 2002 *Clin. Canc. Res.* 8:2024; Gurumurthy et al., 2001 *Cancer Metastas. Rev.* 20:225; Kanduc et al., 2002 *Int. J. Oncol.* 21:165). Typically an apoptosis signaling polypeptide sequence comprises all or a portion of, or is derived from, a receptor death domain polypeptide, for instance, FADD (e.g., Genbank Acc. Nos. U24231, U43184, AF009616, AF009617, NM_012115), TRADD (e.g., Genbank Acc. No. NM_003789), RAIDD (e.g., Genbank Acc. No. U87229), CD95 (FAS/Apo-1; e.g., Genbank Acc. Nos. X89101, NM_003824, AF344850, AF344856), TNF-α-receptor-1 (TNFR1, e.g., Genbank Acc. Nos. S63368, AF040257), DR5 (e.g., Genbank Acc. No. AF020501, AF016268, AF012535), an ITIM domain (e.g., Genbank Acc. Nos. AF081675, BC015731, NM_006840, NM_006844, NM_006847, XM_017977; see, e.g., Billadeau et al., 2002 *J. Clin. Invest.* 109:161), an ITAM domain (e.g., Genbank Acc. Nos. NM_005843, NM_003473, BC030586; see, e.g., Billadeau et al., 2002), or other apoptosis-associated receptor death domain polypeptides known to the art, for example, TNFR2 (e.g., Genbank Acc. No. L49431, L49432), caspase/procaspase-3 (e.g., Genbank Acc. No. XM_54686), caspase/procaspase-8 (e.g., AF380342, NM_004208, NM_001228, NM_033355, NM_033356, NM_033357, NM_033358), caspase/procaspase-2 (e.g., Genbank Acc. No. AF314174, AF314175), etc.

[0130] Cells in a biological sample that are suspected of undergoing apoptosis may be examined for morphological, permeability or other changes that are indicative of an apoptotic state. For example by way of illustration and not limitation, apoptosis in many cell types may cause altered morphological appearance such as plasma membrane blebbing, cell shape change, loss of substrate adhesion properties or other morphological changes that can be readily detected by a person having ordinary skill in the art, for example by using light microscopy. As another example, cells undergoing apoptosis may exhibit fragmentation and disintegration of chromosomes, which may be apparent by microscopy and/or through the use of DNA-specific or chromatin-specific dyes that are known in the art, including fluorescent dyes. Such cells may also exhibit altered plasma membrane permeability properties as may be readily detected through the use of vital dyes (e.g., propidium iodide, trypan blue) or by the detection of lactate dehydrogenase leakage into the extracellular milieu. These and other means for detecting apoptotic cells by morphologic criteria, altered plasma membrane permeability and related changes will be apparent to those familiar with the art.

[0131] In another embodiment of the invention wherein a binding domain-immunoglobulin fusion protein that is expressed at a cell surface comprises a plasma membrane anchor domain having a transmembrane domain and a cytoplasmic tail that comprises an apoptosis signaling polypeptide, cells in a biological sample may be assayed for translocation of cell membrane phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane, which may be detected, for example, by measuring outer leaflet binding by the PS-specific protein annexin. (Martin et al., *J. Exp. Med.* 182:1545, 1995; Fadok et al., *J. Immunol.* 148:2207, 1992.) In still other related embodiments of the invention, including embodiments wherein the binding domain-immunoglobulin fusion protein is expressed at the cell surface and comprises a plasma membrane anchor domain having an apoptosis signaling polypeptide and also including embodiments wherein the binding domain-immunoglobulin fusion protein is a soluble protein that lacks a membrane anchor domain and that is capable of inducing apoptosis, a cellular response to an apoptogen is determined by an assay for induction of specific protease activity in any member of a family of apoptosis-activated proteases known as the caspases (see, e.g., Green et al., 1998 *Science* 281:1309). Those having ordinary skill in the art will be readily familiar with methods for determining caspase activity, for example by determination of caspase-mediated cleavage of specifically recognized protein substrates. These substrates may include, for example, poly-(ADP-ribose) polymerase (PARP) or other naturally occurring or synthetic peptides and proteins cleaved by caspases that are known in the art (see, e.g., Ellerby et al., 1997 *J. Neurosci.* 17:6165). The synthetic peptide Z-Tyr-Val-Ala-Asp-AFC (SEQ ID NO:______;), wherein "Z" indicates a benzoyl carbonyl moiety and AFC indicates 7-amino-4-trifluoromethylcoumarin (Kluck et al., 1997 *Science* 275:1132; Nicholson et al., 1995 *Nature* 376:37), is one such substrate. Other non-limiting examples of substrates include nuclear proteins such as U1-70 kDa and DNA-PKcs (Rosen and Casciola-Rosen, 1997 *J. Cell. Biochem.* 64:50; Cohen, 1997 *Biochem. J.* 326:1). Cellular apoptosis may also be detected by determination of cytochrome c that has escaped from mitochondria in apoptotic cells (e.g., Liu et al., *Cell* 86:147, 1996). Such detection of cytochrome c may be performed spectrophotometrically, immunochemically or by other well established methods for determining the presence of a specific protein. Persons having ordinary skill in the art will readily appreciate that there may be other suitable techniques for quantifying apoptosis.

[0132] Once a binding domain-immunoglobulin fusion protein as provided herein has been designed, DNAs encoding the polypeptide may be synthesized via oligonucleotide synthesis as described, for example, in Sinha et al., *Nucleic Acids Res.,* 12, 4539-4557 (1984); assembled via PCR as described, for example in Innis, Ed., *PCR Protocols,* Academic Press (1990) and also in Better et al. *J. Biol. Chem.* 267, 16712-16118 (1992); cloned and expressed via standard procedures as described, for example, in Ausubel et al., Eds., *Current Protocols in Molecular Biology,* John Wiley & Sons, New York (1989) and also in Robinson et al., *Hum. Antibod. Hybridomas,* 2, 84-93 (1991); and tested for specific antigen binding activity, as described, for example, in Harlow et al., Eds., *Antibodies: A Laboratory Manual,* Chapter 14, Cold Spring Harbor Laboratory, Cold Spring Harbor (1988) and Munson et al., *Anal. Biochem.,* 107, 220-239 (1980).

[0133] The preparation of single polypeptide chain binding molecules of the Fv region, single-chain Fv molecules, is described in U.S. Pat. No. 4,946,778, which is incorporated herein by reference. In the present invention, single-chain Fv-like molecules are synthesized by encoding a first variable region of the heavy or light chain, followed by one or more linkers to the variable region of the corresponding light or heavy chain, respectively. The selection of appropriate linker(s) between the two variable regions is described in U.S. Pat. No. 4,946,778 (see also, e.g., Huston et al., 1993

Int. Rev. Immunol. 10:195). An exemplary linker described herein is (Gly-Gly-Gly-Gly-Ser)$_3$. The linker is used to convert the naturally aggregated but chemically separate heavy and light chains into the amino terminal antigen binding portion of a single polypeptide chain, wherein this antigen binding portion will fold into a structure similar to the original structure made of two polypeptide chains and thus retain the ability to bind to the antigen of interest. The nucleotide sequences encoding the variable regions of the heavy and light chains, joined by a sequence encoding a linker, are joined to a nucleotide sequence encoding antibody constant regions. The constant regions are those which permit the resulting polypeptide to form interchain disulfide bonds to form a dimer, and which contain desired effector functions, such as the ability to mediate antibody-dependent cellular cytotoxicity (ADCC). For an immunoglobulin-like molecule of the invention which is intended for use in humans, the constant regions will typically be substantially human to minimize a potential anti-human immune response and to provide appropriate effector functions. Manipulation of sequences encoding antibody constant regions is described in the PCT publication of Morrison and Oi, WO 89/07142, which is incorporated herein by reference. In preferred embodiments, the CH1 domain is deleted and the carboxyl end of the binding domain polypeptide (e.g., an immunoglobulin variable region polypeptide) is joined to the amino terminus of CH2 via a hinge region polypeptide as provided herein.

[0134] As described above, the present invention provides recombinant expression constructs capable of directing the expression of binding domain-immunoglobulin fusion proteins as provided herein. The amino acids, which occur in the various amino acid sequences referred to herein, are identified according to their well known three-letter or single-letter abbreviations. The nucleotides, which occur in the various DNA sequences or fragments thereof referred herein, are designated with the standard single letter designations used routinely in the art. A given amino acid sequence may also encompass similar amino acid sequences having only minor changes, for example by way of illustration and not limitation, covalent chemical modifications, insertions, deletions and substitutions, which may further include conservative substitutions. Amino acid sequences that are similar to one another may share substantial regions of sequence homology. In like fashion, nucleotide sequences may encompass substantially similar nucleotide sequences having only minor changes, for example by way of illustration and not limitation, covalent chemical modifications, insertions, deletions and substitutions, which may further include silent mutations owing to degeneracy of the genetic code. Nucleotide sequences that are similar to one another may share substantial regions of sequence homology.

[0135] The presence of a malignant condition in a subject refers to the presence of dysplastic, cancerous and/or transformed cells in the subject, including, for example neoplastic, tumor, non-contact inhibited or oncogenically transformed cells, or the like (e.g., melanoma, carcinomas such as adenocarcinoma, squamous cell carcinoma, small cell carcinoma, oat cell carcinoma, etc., sarcomas such as chondrosarcoma, osteosarcoma, etc.) which are known to the art and for which criteria for diagnosis and classification are established. In preferred embodiments contemplated by the present invention, for example, such cancer cells are malignant hematopoietic cells, such as transformed cells of lymphoid lineage and in particular, B-cell lymphomas and the like; cancer cells may in certain preferred embodiments also be epithelial cells such as carcinoma cells. The invention also contemplates B-cell disorders, which may include certain malignant conditions that affect B-cells (e.g., B-cell lymphoma) but which is not intended to be so limited, and which is also intended to encompass autoimmune diseases and in particular, diseases, disorders and conditions that are characterized by autoantibody production.

[0136] Autoantibodies are antibodies that react with self antigens. Autoantibodies are detected in several autoimmune diseases (i.e., a disease, disorder or condition wherein a host immune system generates an inappropriate anti-"self" immune reaction) where they are involved in disease activity. The current treatments for these autoimmune diseases are immunosuppressive drugs that require continuing administration, lack specificity, and cause significant side effects. New approaches that can eliminate autoantibody production with minimal toxicity will address an unmet medical need for a spectrum of diseases that affect many people. The subject invention binding domain-immunoglobulin fusion protein is designed for improved penetration into lymphoid tissues. Depletion of B lymphocytes interrupts the autoantibody production cycle, and allows the immune system to reset as new B lymphocytes are produced from precursors in the bone marrow.

[0137] A number of diseases have been identified for which beneficial effects are believed, according to non-limiting theory, to result from B cell depletion therapy; a brief description of several exemplars of these diseases follows.

[0138] Autoimmune thyroid disease includes Graves' disease and Hashimoto's thyroiditis. In the United States alone, there are about 20 million people who have some form of autoimmune thyroid disease. Autoimmune thyroid disease results from the production of autoantibodies that either stimulate the thyroid to cause hyperthyroidism (Graves' disease) or destroy the thyroid to cause hypothyroidism (Hashimoto's thyroiditis). Stimulation of the thyroid is caused by autoantibodies that bind and activate the thyroid stimulating hormone (TSH) receptor. Destruction of the thyroid is caused by autoantibodies that react with other thyroid antigens.

[0139] Current therapy for Graves' disease includes surgery, radioactive iodine, or antithyroid drug therapy. Radioactive iodine is widely used, since antithyroid medications have significant side effects and disease recurrence is high. Surgery is reserved for patients with large goiters or where there is a need for very rapid normalization of thyroid function. There are no therapies that target the production of autoantibodies responsible for stimulating the TSH receptor. Current therapy for Hashimoto's thyroiditis is levothyroxine sodium, and therapy is usually lifelong because of the low likelihood of remission. Suppressive therapy has been shown to shrink goiters in Hashimoto's thryoiditis, but no therapies that reduce autoantibody production to target the disease mechanism are known.

[0140] Rheumatoid arthritis (RA) is a chronic disease characterized by inflamation of the joints, leading to swelling, pain, and loss of function. RA effects an estimated 2.5 million people in the United States. RA is caused by a combination of events including an initial infection or injury,

an abnormal immune response, and genetic factors. While autoreactive T cells and B cells are present in RA, the detection of high levels of antibodies that collect in the joints, called rheumatoid factor, is used in the diagnosis of RA. Current therapy for RA includes many medications for managing pain and slowing the progression of the disease. No therapy has been found that can cure the disease. Medications include nonsteroidal antiinflamatory drugs (NSAIDS), and disease modifying antirheumatic drugs (DMARDS). NSAIDS are effective in benign disease, but fail to prevent the progression to joint destruction and debility in severe RA. Both NSAIDS and DMARDS are associated with significant side effects. Only one new DMARD, Leflunomide, has been approved in over 10 years. Leflunomide blocks production of autoantibodies, reduces inflamation, and slows progression of RA. However, this drug also causes severe side effects including nausea, diarrhea, hair loss, rash, and liver injury.

[0141] Systemic Lupus Erythematosus (SLE) is an autoimmune disease caused by recurrent injuries to blood vessels in multiple organs, including the kidney, skin, and joints. SLE effects over 500,000 people in the United States. In patients with SLE, a faulty interaction between T cells and B cells results in the production of autoantibodies that attack the cell nucleus. These include anti-double stranded DNA and anti-Sm antibodies. Autoantibodies that bind phospholipids are also found in about half of SLE patients, and are responsible for blood vessel damage and low blood counts. Immune complexes accumulate the kidneys, blood vessels, and joints of SLE patients, where they cause inflamation and tissue damage. No treatment for SLE has been found to cure the disease. NSAIDS and DMARDS are used for therapy depending upon the severity of the disease. Plasmapheresis with plasma exchange to remove autoantibodies can cause temporary improvement in SLE patients. There is general agreement that autoantibodies are responsible for SLE, so new therapies that deplete the B cell lineage, allowing the immune system to reset as new B cells are generated from precursors, offer hope for long lasting benefit in SLE patients.

[0142] Sjogrens syndrome is an autoimmune disease characterized by destruction of the body's moisture producing glands. Sjogrens syndrome is one of the most prevelant autoimmune disorders, striking up to 4 million people in the United States. About half of people with Sjogren's also have a connective tissue disease, such as rheumatoid arthritis, while the other half have primary Sjogren's with no other concurrent autoimmune disease. Autoantibodies, including anti-nuclear antibodies, rheumatoid factor, anti-fodrin, and anti-muscarinic receptor are often present in patients with Sjogrens syndrome. Conventional therapy includes corticosteroids.

[0143] Immune Thrombocytopenic purpura (ITP) is caused by autoantibodies that bind to blood platelets and cause their destruction. Some cases of ITP are caused by drugs, and others are associated with infection, pregnancy, or autoimmune disease such as SLE. About half of all cases are classified as "idiopathic", meaning the cause is unknown. The treatment of ITP is determined by the severity of the symptoms. In some cases, no therapy is needed. In most cases, immunosuppressive drugs, including corticosteroids or intravenous infusions of immune globulin to deplete T cells. Another treatment that usually results in an increased number of platelets is removal of the spleen, the organ that destroys antibody-coated platelets. More potent immunosuppressive drugs, including cyclosporine, cyclophosphamide, or azathioprine are used for patients with severe cases. Removal of autoantibodies by passage of patients' plasma over a Protein A column is used as a second line treatment in patients with severe disease.

[0144] Multiple Sclerosis (MS) is an autoimmune disease characterized by inflamation of the central nervous system and destruction of myelin, which insulates nerve cell fibers in the brain, spinal cord, and body. Although the cause of MS is unknown, it is widely believed that autoimmune T cells are primary contributors to the pathogenesis of the disease. However, high levels of antibodies are present in the cerebral spinal fluid of patients with MS, and some theories predict that the B cell response leading to antibody production is important for mediating the disease. No B cell depletion therapies have been studies in patients with MS. There is no cure for MS. Current therapy is corticosteroids, which can reduce the duration and severity of attacks, but do not affect the course of MS over time. New biotechnology interferon (IFN) therapies for MS have recently been approved.

[0145] Myasthenia Gravis (MG) is a chronic autoimmune neuromuscular disorder that is characterized by weakness of the voluntary muscle groups. MG effects about 40,000 people in the United States. MG is caused by autoantibodies that bind to acetylcholine receptors expressed at neuromuscular junctions. The autoantibodies reduce or block acetylcholine receptors, preventing the transmission of signals from nerves to muscles. There is no known cure for MG. Common treatments include immunosuppression with corticosteroids, cyclosporine, cyclophosphamide, or azathioprine. Surgical removal of the thymus is often used to blunt the autoimmune response. Plasmapheresis, used to reduce autoantibody levels in the blood, is effective in MG, but is short-lived because the production of autoantibodies continues. Plasmapheresis is usually reserved for severe muscle weakness prior to surgery.

[0146] Psoriasis effects approximately five million people. Autoimmune inflamation in the skin. Psoriasis associated with arthritis in 30% (psoriatic arthritis). Many treatments, including steroids, UV light retenoids, vitamin D derivatives, cyclosporine, methotrexate.

[0147] Scleroderma is a chronic autoimmune disease of the connective tissue that is also known as systemic sclerosis. Scleroderma is characterized by an overproduction of collagen, resulting in a thickening of the skin. Approximately 300,000 people in the United States have scleroderma.

[0148] Inflamatory Bowel Disease including Crohn's disease and Ulcerative colitis, are autoimmune diseases of the digestive system.

[0149] The present invention further relates to constructs encoding binding domain-immunoglobulin fusion proteins, and in particular to methods for administering recombinant constructs encoding such proteins that may be expressed, for example, as fragments, analogs and derivatives of such polypeptides. The terms "fragment," "derivative" and "analog" when referring to binding domain-immunoglobulin fusion polypeptides or fusion proteins, refers to any binding

domain-immunoglobulin fusion polypeptide or fusion protein that retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active binding domain-immunoglobulin fusion polypeptide.

[0150] A fragment, derivative or analog of an binding domain-immunoglobulin fusion polypeptide or fusion protein, including binding domain-immunoglobulin fusion polypeptides or fusion proteins encoded by the cDNAs referred to herein, may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which additional amino acids are fused to the binding domain-immunoglobulin fusion polypeptide, including amino acids that are employed for detection or specific functional alteration of the binding domain-immunoglobulin fusion polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

[0151] The polypeptides of the present invention include binding domain-immunoglobulin fusion polypeptides and fusion proteins having binding domain polypeptide amino acid sequences that are identical or similar to sequences known in the art, or fragments or portions thereof. For example by way of illustration and not limitation, a human CD154 molecule extracellular domain [SEQ ID NO:______] is contemplated for use according to the instant invention, as are polypeptides having at least 70% similarity (preferably a 70% identity) and more preferably 90% similarity (more preferably a 90% identity) to the reported polypeptide and still more preferably a 95% similarity (still more preferably a 95% identity) to the reported polypeptides and to portions of such polypeptides, wherein such portions of a binding domain-immunoglobulin fusion polypeptide generally contain at least 30 amino acids and more preferably at least 50 amino acids. Extracellular domains include portions of a cell surface molecule, and in particularly preferred embodiments cell surface molecules that are integral membrane proteins or that comprise a plasma membrane spanning transmembrane domain, that extend beyond the outer leaflet of the plasma membrane phospholipid bilayer when the molecule is expressed at a cell surface, preferably in a manner that exposes the extracellular domain portion of such a molecule to the external environment of the cell, also known as the extracellular milieu. Methods for determining whether a portion of a cell surface molecule comprises an extracellular domain are well known to the art and include experimental determination (e.g., direct or indirect labeling of the molecule, evaluation of whether the molecule can be structurally altered by agents to which the plasma membrane is not permeable such as proteolytic or lipolytic enzymes) or topological prediction based on the structure of the molecule (e.g., analysis of the amino acid sequence of a polypeptide) or other methodologies.

[0152] As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and conserved amino acid substitutes thereto of the polypeptide to the sequence of a second polypeptide. Fragments or portions of the nucleic acids encoding polypeptides of the present invention may be used to synthesize full-length nucleic acids of the present invention. As used herein, "% identity" refers to the percentage of identical amino acids situated at corresponding amino acid residue positions when two or more polypeptide are aligned and their sequences analyzed using a gapped BLAST algorithm (e.g., Altschul et al., 1997 *Nucl. Ac. Res.* 25:3389) which weights sequence gaps and sequence mismatches according to the default weightings provided by the National Institutes of Health/NCBI database (Bethesda, Md; see www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast).

[0153] The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally occurring nucleic acid or polypeptide present in a living animal is not isolated, but the same nucleic acid or polypeptide, separated from some or all of the co-existing materials in the natural system, is isolated. Such nucleic acids could be part of a vector and/or such nucleic acids or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

[0154] The term "gene" means the segment of DNA involved in producing a polypeptide chain; it includes regions preceding and following the coding region "leader and trailer" as well as intervening sequences (introns) between individual coding segments (exons).

[0155] As described herein, the invention provides binding domain-immunoglobulin fusion proteins encoded by nucleic acids that have the binding domain coding sequence fused in frame to an additional immunoglobulin domain encoding sequence to provide for expression of a binding domain polypeptide sequence fused to an additional functional polypeptide sequence that permits, for example by way of illustration and not limitation, detection, functional alteration, isolation and/or purification of the fusion protein. Such fusion proteins may permit functional alteration of a binding domain by containing additional immunoglobulin-derived polypeptide sequences that influence behavior of the fusion product, for example (and as described above) by reducing the availability of sulfhydryl groups for participation in disulfide bond formation, and by conferring the ability to potentiate ADCC and/or CDC.

[0156] Modification of the polypeptide may be effected by any means known to those of skill in this art. The preferred methods herein rely on modification of DNA encoding the fusion protein and expression of the modified DNA. DNA encoding one of the binding domain-immunoglobulin fusions discussed above may be mutagenized using standard methodologies, including those described below. For example, cysteine residues that may otherwise facilitate multimer formation or promote particular molecular conformations can be deleted from a polypeptide or replaced, e.g., cysteine residues that are responsible for aggregate formation. If necessary, the identity of cysteine residues that contribute to aggregate formation may be determined empirically, by deleting and/or replacing a cysteine residue and ascertaining whether the resulting protein aggregates in solutions containing physiologically acceptable buffers and salts. In addition, fragments of binding domain-immunoglobulin fusions may be constructed and used. As noted above,

the counterreceptor/ligand binding domains for many candidate binding domain-immunoglobulin fusion have been delineated, such that one having ordinary skill in the art may readily select appropriate polypeptide domains for inclusion in the encoded products of the instant expression constructs.

[0157] Conservative substitutions of amino acids are well-known and may be made generally without altering the biological activity of the resulting binding domain-immunoglobulin fusion protein molecule. For example, such substitutions are generally made by interchanging within the groups of polar residues, charged residues, hydrophobic residues, small residues, and the like. If necessary, such substitutions may be determined empirically merely by testing the resulting modified protein for the ability to bind to the appropriate cell surface receptors in in vitro biological assays, or to bind to appropriate antigens or desired target molecules.

[0158] The present invention further relates to nucleic acids which hybridize to binding domain-immunoglobulin fusion protein encoding polynucleotide sequences as provided herein, or their complements, as will be readily apparent to those familiar with the art, if there is at least 70%, preferably 80-85%, more preferably at least 90%, and still more preferably at least 95%, 96%, 97%, 98% or 99% identity between the sequences. The present invention particularly relates to nucleic acids which hybridize under stringent conditions to the binding domain-immunoglobulin fusion encoding nucleic acids referred to herein. As used herein, the term "stringent conditions" means hybridization will occur only if there is at least 90-95% and preferably at least 97% identity between the sequences. The nucleic acids which hybridize to binding domain-immunoglobulin fusion encoding nucleic acids referred to herein, in preferred embodiments, encode polypeptides which retain substantially the same biological function or activity as the binding domain-immunoglobulin fusion polypeptides encoded by the cDNAs of the references cited herein.

[0159] As used herein, to "hybridize" under conditions of a specified stringency is used to describe the stability of hybrids formed between two single-stranded nucleic acid molecules. Stringency of hybridization is typically expressed in conditions of ionic strength and temperature at which such hybrids are annealed and washed. Typically "high", "medium" and "low" stringency encompass the following conditions or equivalent conditions thereto: high stringency: 0.1xSSPE or SSC, 0.1% SDS, 65° C.; medium stringency: 0.2xSSPE or SSC, 0.1% SDS, 50° C.; and low stringency: 1.0xSSPE or SSC, 0.1% SDS, 50° C. As known to those having ordinary skill in the art, variations in stringency of hybridization conditions may be achieved by altering the time, temperature and/or concentration of the solutions used for prehybridization, hybridization and wash steps, and suitable conditions may also depend in part on the particular nucleotide sequences of the probe used, and of the blotted, proband nucleic acid sample. Accordingly, it will be appreciated that suitably stringent conditions can be readily selected without undue experimentation where a desired selectivity of the probe is identified, based on its ability to hybridize to one or more certain proband sequences while not hybridizing to certain other proband sequences.

[0160] The nucleic acids of the present invention, also referred to herein as polynucleotides, may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. A coding sequence which encodes an binding domain-immunoglobulin fusion polypeptide for use according to the invention may be identical to the coding sequence known in the art for any given binding domain-immunoglobulin fusion, or may be a different coding sequence, which, as a result of the redundancy or degeneracy of the genetic code, encodes the same binding domain-immunoglobulin fusion polypeptide.

[0161] The nucleic acids which encode binding domain-immunoglobulin fusion polypeptides for use according to the invention may include, but are not limited to: only the coding sequence for the binding domain-immunoglobulin fusion polypeptide; the coding sequence for the binding domain-immunoglobulin fusion polypeptide and additional coding sequence; the coding sequence for the binding domain-immunoglobulin fusion polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequences 5' and/or 3' of the coding sequence for the binding domain-immunoglobulin fusion polypeptide, which for example may further include but need not be limited to one or more regulatory nucleic acid sequences that may be a regulated or regulatable promoter, enhancer, other transcription regulatory sequence, repressor binding sequence, translation regulatory sequence or any other regulatory nucleic acid sequence. Thus, the term "nucleic acid encoding" or "polynucleotide encoding" a binding domain-immunoglobulin fusion protein encompasses a nucleic acid which includes only coding sequence for a binding domain-immunoglobulin fusion polypeptide as well as a nucleic acid which includes additional coding and/or non-coding sequence(s).

[0162] Nucleic acids and oligonucleotides for use as described herein can be synthesized by any method known to those of skill in this art (see, e.g., WO 93/01286, U.S. application Ser. No. 07/723,454; U.S. Pat. No. 5,218,088; U.S. Pat. No. 5,175,269; U.S. Pat. No. 5,109,124). Identification of oligonucleotides and nucleic acid sequences for use in the present invention involves methods well known in the art. For example, the desirable properties, lengths and other characteristics of useful oligonucleotides are well known. In certain embodiments, synthetic oligonucleotides and nucleic acid sequences may be designed that resist degradation by endogenous host cell nucleolytic enzymes by containing such linkages as: phosphorothioate, methylphosphonate, sulfone, sulfate, ketyl, phosphorodithioate, phosphoramidate, phosphate esters, and other such linkages that have proven useful in antisense applications (see, e.g., Agrwal et al., *Tetrehedron Lett.* 28:3539-3542 (1987); Miller et al., *J. Am. Chem. Soc.* 93:6657-6665 (1971); Stec et al., *Tetrehedron Lett.* 26:2191-2194 (1985); Moody et al., *Nucl. Acids Res.* 12:4769-4782 (1989); Uznanski et al., *Nucl. Acids Res.* (1989); Letsinger et al., *Tetrahedron* 40:137-143 (1984); Eckstein, *Annu. Rev. Biochem.* 54:367-402 (1985); Eckstein, *Trends Biol. Sci.* 14:97-100 (1989); Stein In: *Oligodeoxynucleotides. Antisense Inhibitors of Gene Expression,* Cohen, Ed, Macmillan Press, London, pp. 97-117 (1989); Jager et al., *Biochemistry* 27:7237-7246 (1988)).

[0163] In one embodiment, the present invention provides truncated components (e.g., binding domain polypeptide, hinge region polypeptide, linker, etc.) for use in a binding domain-immunoglobulin fusion protein, and in another embodiment the invention provides nucleic acids encoding a binding domain-immunoglobulin fusion protein having such truncated components. A truncated molecule may be any molecule that comprises less than a full length version of the molecule. Truncated molecules provided by the present invention may include truncated biological polymers, and in preferred embodiments of the invention such truncated molecules may be truncated nucleic acid molecules or truncated polypeptides. Truncated nucleic acid molecules have less than the full length nucleotide sequence of a known or described nucleic acid molecule, where such a known or described nucleic acid molecule may be a naturally occurring, a synthetic or a recombinant nucleic acid molecule, so long as one skilled in the art would regard it as a full length molecule. Thus, for example, truncated nucleic acid molecules that correspond to a gene sequence contain less than the full length gene where the gene comprises coding and non-coding sequences, promoters, enhancers and other regulatory sequences, flanking sequences and the like, and other functional and non-functional sequences that are recognized as part of the gene. In another example, truncated nucleic acid molecules that correspond to a mRNA sequence contain less than the full length mRNA transcript, which may include various translated and non-translated regions as well as other functional and non-functional sequences.

[0164] In other preferred embodiments, truncated molecules are polypeptides that comprise less than the full length amino acid sequence of a particular protein or polypeptide component. As used herein "deletion" has its common meaning as understood by those familiar with the art, and may refer to molecules that lack one or more of a portion of a sequence from either terminus or from a non-terminal region, relative to a corresponding full length molecule, for example, as in the case of truncated molecules provided herein. Truncated molecules that are linear biological polymers such as nucleic acid molecules or polypeptides may have one or more of a deletion from either terminus of the molecule or a deletion from a non-terminal region of the molecule, where such deletions may be deletions of 1-1500 contiguous nucleotide or amino acid residues, preferably 1-500 contiguous nucleotide or amino acid residues and more preferably 1-300 contiguous nucleotide or amino acid residues, including deletions of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31-40, 41-50, 51-74, 75-100, 101-150, 151-200, 201-250 or 251-299 contiguous nucleotide or amino acid residues. In certain particularly preferred embodiments truncated nucleic acid molecules may have a deletion of 270-330 contiguous nucleotides. In certain other particularly preferred embodiments truncated polypeptide molecules may have a deletion of 80-140 contiguous amino acids.

[0165] The present invention further relates to variants of the herein referenced nucleic acids which encode fragments, analogs and or derivatives of a binding domain-immunoglobulin fusion polypeptide. The variants of the nucleic acids encoding binding domain-immunoglobulin fusion may be naturally occurring allelic variants of the nucleic acids or non-naturally occurring variants. As is known in the art, an allelic variant is an alternate form of a nucleic acid sequence which may have at least one of a substitution, a deletion or an addition of one or more nucleotides, any of which does not substantially alter the function of the encoded binding domain-immunoglobulin fusion polypeptide.

[0166] Variants and derivatives of binding domain-immunoglobulin fusion may be obtained by mutations of nucleotide sequences encoding binding domain-immunoglobulin fusion polypeptides or any portion thereof. Alterations of the native amino acid sequence may be accomplished by any of a number of conventional methods. Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

[0167] Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene wherein predetermined codons can be altered by substitution, deletion or insertion. Exemplary methods of making such alterations are disclosed by Walder et al. (*Gene* 42:133, 1986); Bauer et al. (*Gene* 37:73, 1985); Craik (*BioTechniques,* Jan. 12-19, 1985); Smith et al. (*Genetic Engineering: Principles and Methods BioTechniques,* Jan. 12-19, 1985); Smith et al. (*Genetic Engineering: Principles and Methods,* Plenum Press, 1981); Kunkel (*Proc. Natl. Acad. Sci. USA* 82:488, 1985); Kunkel et al. (*Methods in Enzymol.* 154:367, 1987); and U.S. Pat. Nos. 4,518,584 and 4,737,462.

[0168] As an example, modification of DNA may be performed by site-directed mutagenesis of DNA encoding the protein combined with the use of DNA amplification methods using primers to introduce and amplify alterations in the DNA template, such as PCR splicing by overlap extension (SOE). Site-directed mutagenesis is typically effected using a phage vector that has single- and double-stranded forms, such as M13 phage vectors, which are well-known and commercially available. Other suitable vectors that contain a single-stranded phage origin of replication may be used (see, e.g., Veira et al., *Meth. Enzymol.* 15:3, 1987). In general, site-directed mutagenesis is performed by preparing a single-stranded vector that encodes the protein of interest (e.g., all or a component portion of a given binding domain-immunoglobulin fusion protein). An oligonucleotide primer that contains the desired mutation within a region of homology to the DNA in the single-stranded vector is annealed to the vector followed by addition of a DNA polymerase, such as *E. coli* DNA polymerase I (Klenow fragment), which uses the double stranded region as a primer to produce a heteroduplex in which one strand encodes the altered sequence and the other the original sequence. The heteroduplex is introduced into appropriate bacterial cells and clones that include the desired mutation are selected. The resulting altered DNA molecules may be expressed recombinantly in appropriate host cells to produce the modified protein.

[0169] Equivalent DNA constructs that encode various additions or substitutions of amino acid residues or sequences, or deletions of terminal or internal residues or sequences not needed for biological activity are also encompassed by the invention. For example, and as discussed

above, sequences encoding Cys residues that are not desirable or essential for biological activity can be altered to cause the Cys residues to be deleted or replaced with other amino acids, preventing formation of incorrect intramolecular disulfide bridges upon renaturation.

[0170] Host organisms include those organisms in which recombinant production of binding domain-immunoglobulin fusion products encoded by the recombinant constructs of the present invention may occur, such as bacteria (for example, *E. coli*), yeast (for example, *Saccharomyces cerevisiae* and *Pichia pastoris*), insect cells and mammals, including in vitro and in vivo expression. Host organisms thus may include organisms for the construction, propagation, expression or other steps in the production of the compositions provided herein; hosts also include subjects in which immune responses take place, as described above. Presently preferred host organisms are *E. coli* bacterial strains, inbred murine strains and murine cell lines, and human cells, subjects and cell lines.

[0171] The DNA construct encoding the desired binding domain-immunoglobulin fusion is introduced into a plasmid for expression in an appropriate host. In preferred embodiments, the host is a bacterial host. The sequence encoding the ligand or nucleic acid binding domain is preferably codon-optimized for expression in the particular host. Thus, for example, if a human binding domain-immunoglobulin fusion is expressed in bacteria, the codons would be optimized for bacterial usage. For small coding regions, the gene can be synthesized as a single oligonucleotide. For larger proteins, splicing of multiple oligonucleotides, mutagenesis, or other techniques known to those in the art may be used. The sequences of nucleotides in the plasmids that are regulatory regions, such as promoters and operators, are operationally associated with one another for transcription. The sequence of nucleotides encoding a binding domain-immunoglobulin fusion protein may also include DNA encoding a secretion signal, whereby the resulting peptide is a precursor protein. The resulting processed protein may be recovered from the periplasmic space or the fermentation medium.

[0172] In preferred embodiments, the DNA plasmids also include a transcription terminator sequence. As used herein, a "transcription terminator region" is a sequence that signals transcription termination. The entire transcription terminator may be obtained from a protein-encoding gene, which may be the same or different from the inserted binding domain-immunoglobulin fusion encoding gene or the source of the promoter. Transcription terminators are optional components of the expression systems herein, but are employed in preferred embodiments.

[0173] The plasmids used herein include a promoter in operative association with the DNA encoding the protein or polypeptide of interest and are designed for expression of proteins in a suitable host as described above (e.g., bacterial, murine or human) depending upon the desired use of the plasmid (e.g., administration of a vaccine containing binding domain-immunoglobulin fusion encoding sequences). Suitable promoters for expression of proteins and polypeptides herein are widely available and are well known in the art. Inducible promoters or constitutive promoters that are linked to regulatory regions are preferred. Such promoters include, but are not limited to, the T7 phage promoter and other T7-like phage promoters, such as the T3, T5 and SP6 promoters, the trp, lpp, and lac promoters, such as the lacUV5, from *E. coli;* the P10 or polyhedrin gene promoter of baculovirus/insect cell expression systems (see, e.g., U.S. Pat. Nos. 5,243,041, 5,242,687, 5,266,317, 4,745,051, and 5,169,784) and inducible promoters from other eukaryotic expression systems. For expression of the proteins such promoters are inserted in a plasmid in operative linkage with a control region such as the lac operon.

[0174] Preferred promoter regions are those that are inducible and functional in *E. coli.* Examples of suitable inducible promoters and promoter regions include, but are not limited to: the *E. coli* lac operator responsive to isopropyl β-D-thiogalactopyranoside (IPTG; see Nakamura et al., *Cell* 18:1109-1117, 1979); the metallothionein promoter metal-regulatory-elements responsive to heavy-metal (e.g., zinc) induction (see, e.g., U.S. Pat. No. 4,870,009 to Evans et al.); the phage T7lac promoter responsive to IPTG (see, e.g., U.S. Pat. No. 4,952,496; and Studier et al., *Meth. Enzymol.* 185:60-89, 1990) and the TAC promoter.

[0175] The plasmids may optionally include a selectable marker gene or genes that are functional in the host. A selectable marker gene includes any gene that confers a phenotype on bacteria that allows transformed bacterial cells to be identified and selectively grown from among a vast majority of untransformed cells. Suitable selectable marker genes for bacterial hosts, for example, include the ampicillin resistance gene (Amp^r), tetracycline resistance gene (Tc^r) and the kanamycin resistance gene (Kan^r). The kanamycin resistance gene is presently preferred.

[0176] The plasmids may also include DNA encoding a signal for secretion of the operably linked protein. Secretion signals suitable for use are widely available and are well known in the art. Prokaryotic and eukaryotic secretion signals functional in *E. coli* may be employed. The presently preferred secretion signals include, but are not limited to, those encoded by the following *E. coli* genes: ompA, ompT, ompF, ompC, beta-lactamase, and alkaline phosphatase, and the like (von Heijne, *J. Mol. Biol* 184:99-105, 1985). In addition, the bacterial pelB gene secretion signal (Lei et al., *J. Bacteriol.* 169:4379, 1987), the phoA secretion signal, and the cek2 functional in insect cell may be employed. The most preferred secretion signal is the *E. coli* ompA secretion signal. Other prokaryotic and eukaryotic secretion signals known to those of skill in the art may also be employed (see, e.g., von Heijne, *J. Mol. Biol.* 184:99-105, 1985). Using the methods described herein, one of skill in the art can substitute secretion signals that are functional in either yeast, insect or mammalian cells to secrete proteins from those cells.

[0177] Preferred plasmids for transformation of *E. coli* cells include the pET expression vectors (e.g., pET-11a, pET-12a-c, pET-15b; see U.S. Pat. No. 4,952,496; available from Novagen, Madison, Wis.). Other preferred plasmids include the pKK plasmids, particularly pKK 223-3, which contains the tac promoter (Brosius et al., *Proc. Natl. Acad. Sci.* 81:6929, 1984; Ausubel et al., *Current Protocols in Molecular Biology;* U.S. Pat. Nos. 5,122,463, 5,173,403, 5,187,153, 5,204,254, 5,212,058, 5,212,286, 5,215,907, 5,220,013, 5,223,483, and 5,229,279). Plasmid pKK has been modified by replacement of the ampicillin resistance gene with a kanamycin resistance gene. (Available from

Pharmacia; obtained from pUC4K, see, e.g., Vieira et al. (*Gene* 19:259-268, 1982; and U.S. Pat. No. 4,719,179.) Baculovirus vectors, such as pBlueBac (also called pJVETL and derivatives thereof), particularly pBlueBac III (see, e.g., U.S. Pat. Nos. 5,278,050, 5,244,805, 5,243,041, 5,242,687, 5,266,317, 4,745,051, and 5,169,784; available from Invitrogen, San Diego) may also be used for expression of the polypeptides in insect cells. Other plasmids include the pIN-IIIompA plasmids (see U.S. Pat. No. 4,575,013; see also Duffaud et al., *Meth. Enz.* 153:492-507, 1987), such as pIN-IIIompA2.

[0178] Preferably, the DNA molecule is replicated in bacterial cells, preferably in *E. coli*. The preferred DNA molecule also includes a bacterial origin of replication, to ensure the maintenance of the DNA molecule from generation to generation of the bacteria. In this way, large quantities of the DNA molecule can be produced by replication in bacteria. Preferred bacterial origins of replication include, but are not limited to, the f1-ori and col E1 origins of replication. Preferred hosts contain chromosomal copies of DNA encoding T7 RNA polymerase operably linked to an inducible promoter, such as the lacUV promoter (see U.S. Pat. No. 4,952,496). Such hosts include, but are not limited to, lysogens *E. coli* strains HMS174(DE3)pLysS, BL21(DE3)pLysS, HMS174(DE3) and BL21(DE3). Strain BL21(DE3) is preferred. The pLys strains provide low levels of T7 lysozyme, a natural inhibitor of T7 RNA polymerase.

[0179] The DNA molecules provided may also contain a gene coding for a repressor protein. The repressor protein is capable of repressing the transcription of a promoter that contains sequences of nucleotides to which the repressor protein binds. The promoter can be derepressed by altering the physiological conditions of the cell. For example, the alteration can be accomplished by adding to the growth medium a molecule that inhibits the ability to interact with the operator or with regulatory proteins or other regions of the DNA or by altering the temperature of the growth media. Preferred repressor proteins include, but are not limited to the *E. coli* lacd repressor responsive to IPTG induction, the temperature sensitive λ cI857 repressor, and the like. The *E. coli* lacI repressor is preferred.

[0180] In general, recombinant constructs of the subject invention will also contain elements necessary for transcription and translation. In particular, such elements are preferred where the recombinant expression construct containing nucleic acid sequences encoding binding domain-immunoglobulin fusion proteins is intended for expression in a host cell or organism. In certain embodiments of the present invention, cell type preferred or cell type specific expression of a cell binding domain-immunoglobulin fusion encoding gene may be achieved by placing the gene under regulation of a promoter. The choice of the promoter will depend upon the cell type to be transformed and the degree or type of control desired. Promoters can be constitutive or active and may further be cell type specific, tissue specific, individual cell specific, event specific, temporally specific or inducible. Cell-type specific promoters and event type specific promoters are preferred. Examples of constitutive or nonspecific promoters include the SV40 early promoter (U.S. Pat. No. 5,118,627), the SV40 late promoter (U.S. Pat. No. 5,118,627), CMV early gene promoter (U.S. Pat. No. 5,168,062), and adenovirus promoter. In addition to viral promoters, cellular promoters are also amenable within the context of this invention. In particular, cellular promoters for the so-called housekeeping genes are useful. Viral promoters are preferred, because generally they are stronger promoters than cellular promoters. Promoter regions have been identified in the genes of many eukaryotes including higher eukaryotes, such that suitable promoters for use in a particular host can be readily selected by those skilled in the art.

[0181] Inducible promoters may also be used. These promoters include MMTV LTR (PCT WO 91/13160), inducible by dexamethasone; metallothionein promoter, inducible by heavy metals; and promoters with cAMP response elements, inducible by cAMP. By using an inducible promoter, the nucleic acid sequence encoding a binding domain-immunoglobulin fusion protein may be delivered to a cell by the subject invention expression construct and will remain quiescent until the addition of the inducer. This allows further control on the timing of production of the gene product.

[0182] Event-type specific promoters are active or up-regulated only upon the occurrence of an event, such as tumorigenicity or viral infection. The HIV LTR is a well known example of an event-specific promoter. The promoter is inactive unless the tat gene product is present, which occurs upon viral infection. Some event-type promoters are also tissue-specific.

[0183] Additionally, promoters that are coordinately regulated with a particular cellular gene may be used. For example, promoters of genes that are coordinately expressed may be used when expression of a particular binding domain-immunoglobulin fusion protein-encoding gene is desired in concert with expression of one or more additional endogenous or exogenously introduced genes. This type of promoter is especially useful when one knows the pattern of gene expression relevant to induction of an immune response in a particular tissue of the immune system, so that specific immunocompetent cells within that tissue may be activated or otherwise recruited to participate in the immune response.

[0184] In addition to the promoter, repressor sequences, negative regulators, or tissue-specific silencers may be inserted to reduce non-specific expression of binding domain-immunoglobulin fusion protein encoding genes in certain situations, such as, for example, a host that is transiently immunocompromised as part of a therapeutic strategy. Multiple repressor elements may be inserted in the promoter region. Repression of transcription is independent on the orientation of repressor elements or distance from the promoter. One type of repressor sequence is an insulator sequence. Such sequences inhibit transcription (Dunaway et al., *Mol Cell Biol* 17: 182-9, 1997; Gdula et al., *Proc Natl Acad Sci USA* 93:9378-83, 1996, Chan et al., *J Virol* 70: 5312-28, 1996; Scott and Geyer, *EMBO J* 14:6258-67, 1995; Kalos and Fournier, *Mol Cell Biol* 15:198-207, 1995; Chung et al., *Cell* 74: 505-14, 1993) and will silence background transcription.

[0185] Repressor elements have also been identified in the promoter regions of the genes for type II (cartilage) collagen, choline acetyltransferase, albumin (Hu et al., *J. Cell Growth Differ.* 3(9):577-588, 1992), phosphoglycerate kinase (PGK-2) (Misuno et al., *Gene* 119(2):293-297, 1992), and in the 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase gene. (Lemaigre et al., *Mol. Cell Biol.* 11(2):1099-1106.) Furthermore, the negative regulatory element Tse-1

has been identified in a number of liver specific genes, and has been shown to block cAMP response element- (CRE) mediated induction of gene activation in hepatocytes. (Boshart et al., *Cell* 61(5):905-916, 1990).

[0186] In preferred embodiments, elements that increase the expression of the desired product are incorporated into the construct. Such elements include internal ribosome binding sites (IRES; Wang and Siddiqui, *Curr. Top. Microbiol. Immunol* 203:99, 1995; Ehrenfeld and Semler, *Curr. Top. Microbiol. Immunol.* 203:65, 1995; Rees et al., *Biotechniques* 20:102, 1996; Sugimoto et al., *Biotechnology* 12:694, 1994). IRES increase translation efficiency. As well, other sequences may enhance expression. For some genes, sequences especially at the 5' end inhibit transcription and/or translation. These sequences are usually palindromes that can form hairpin structures. Any such sequences in the nucleic acid to be delivered are generally deleted. Expression levels of the transcript or translated product are assayed to confirm or ascertain which sequences affect expression. Transcript levels may be assayed by any known method, including Northern blot hybridization, RNase probe protection and the like. Protein levels may be assayed by any known method, including ELISA, western blot, immunocytochemistry or other well known techniques.

[0187] Other elements may be incorporated into the binding domain-immunoglobulin fusion protein encoding constructs of the present invention. In preferred embodiments, the construct includes a transcription terminator sequence, including a polyadenylation sequence, splice donor and acceptor sites, and an enhancer. Other elements useful for expression and maintenance of the construct in mammalian cells or other eukaryotic cells may also be incorporated (e.g., origin of replication). Because the constructs are conveniently produced in bacterial cells, elements that are necessary for, or that enhance, propagation in bacteria are incorporated. Such elements include an origin of replication, a selectable marker and the like.

[0188] As provided herein, an additional level of controlling the expression of nucleic acids encoding binding domain-immunoglobulin fusion proteins delivered to cells using the constructs of the invention may be provided by simultaneously delivering two or more differentially regulated nucleic acid constructs. The use of such a multiple nucleic acid construct approach may permit coordinated regulation of an immune response such as, for example, spatiotemporal coordination that depends on the cell type and/or presence of another expressed encoded component. Those familiar with the art will appreciate that multiple levels of regulated gene expression may be achieved in a similar manner by selection of suitable regulatory sequences, including but not limited to promoters, enhancers and other well known gene regulatory elements.

[0189] The present invention also relates to vectors, and to constructs prepared from known vectors that include nucleic acids of the present invention, and in particular to "recombinant expression constructs" that include any nucleic acids encoding binding domain-immunoglobulin fusion proteins and polypeptides according to the invention as provided above; to host cells which are genetically engineered with vectors and or constructs of the invention and to methods of administering expression constructs comprising nucleic acid sequences encoding such binding domain-immunoglobulin fusion polypeptides and fusion proteins of the invention, or fragments or variants thereof, by recombinant techniques. Binding domain-immunoglobulin fusion proteins can be expressed in virtually any host cell under the control of appropriate promoters, depending on the nature of the construct (e.g., type of promoter, as described above), and on the nature of the desired host cell (e.g., whether postmitotic terminally differentiated or actively dividing; e.g., whether the expression construct occurs in host cell as an episome or is integrated into host cell genome). Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., *Molecular Cloning: A Laboratory Manual,* Second Edition, Cold Spring Harbor, N.Y., (1989); as noted above, in particularly preferred embodiments of the invention, recombinant expression is conducted in mammalian cells that have been transfected or transformed with the subject invention recombinant expression construct.

[0190] Typically, the constructs are derived from plasmid vectors. A preferred construct is a modified pNASS vector (Clontech, Palo Alto, Calif.), which has nucleic acid sequences encoding an ampicillin resistance gene, a polyadenylation signal and a T7 promoter site. Other suitable mammalian expression vectors are well known (see, e.g., Ausubel et al., 1995; Sambrook et al., supra; see also, e.g., catalogues from Invitrogen, San Diego, Calif.; Novagen, Madison, Wis.; Pharmacia, Piscataway, N.J.; and others). Presently preferred constructs may be prepared that include a dihydrofolate reductase (DHFR) encoding sequence under suitable regulatory control, for promoting enhanced production levels of the binding domain-immunoglobulin fusion protei, which levels result from gene amplification following application of an appropriate selection agent (e.g., methetrexate).

[0191] Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence, as described above. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences. Thus, for example, the binding domain-immunoglobulin fusion protein encoding nucleic acids as provided herein may be included in any one of a variety of expression vector constructs as a recombinant expression construct for expressing a binding domain-immunoglobulin fusion polypeptide in a host cell. In certain preferred embodiments the constructs are included in formulations that are administered in vivo. Such vectors and constructs include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; yeast plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA, such as vaccinia, adenovirus, fowl pox virus, and pseudorabies, or replication deficient retroviruses as described below. However, any other vector may be used for preparation of a recombinant expression construct, and in preferred embodiments such a vector will be replicable and viable in the host.

[0192] The appropriate DNA sequence(s) may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Standard techniques for cloning, DNA isolation, amplification

and purification, for enzymatic reactions involving DNA ligase, DNA polymerase, restriction endonucleases and the like, and various separation techniques are those known and commonly employed by those skilled in the art. A number of standard techniques are described, for example, in Ausubel et al. (1993 *Current Protocols in Molecular Biology,* Greene Publ. Assoc. Inc. & John Wiley & Sons, Inc., Boston, Mass.); Sambrook et al. (1989 *Molecular Cloning,* Second Ed., Cold Spring Harbor Laboratory, Plainview, N.Y.); Maniatis et al. (1982 Molecular Cloning, Cold Spring Harbor Laboratory, Plainview, N.Y.); Glover (Ed.) (1985 *DNA Cloning Vol. I and II,* IRL Press, Oxford, UK); Hames and Higgins (Eds.), (1985 *Nucleic Acid Hybridization,* IRL Press, Oxford, UK); and elsewhere.

[0193] The DNA sequence in the expression vector is operatively linked to at least one appropriate expression control sequences (e.g., a constitutive promoter or a regulated promoter) to direct mRNA synthesis. Representative examples of such expression control sequences include promoters of eukaryotic cells or their viruses, as described above. Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art, and preparation of certain particularly preferred recombinant expression constructs comprising at least one promoter or regulated promoter operably linked to a nucleic acid encoding an binding domain-immunoglobulin fusion polypeptide is described herein.

[0194] Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Examples including the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

[0195] As provided herein, in certain embodiments the vector may be a viral vector such as a retroviral vector. (Miller et al., 1989 *BioTechniques* 7:980; Coffin and Varmus, 1996 Retroviruses, *Cold Spring Harbor Laboratory Press,* NY.) For example, retroviruses from which the retroviral plasmid vectors may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus.

[0196] Retroviruses are RNA viruses which can replicate and integrate into the genome of a host cell via a DNA intermediate. This DNA intermediate, or provirus, may be stably integrated into the host cell DNA. According to certain embodiments of the present invention, an expression construct may comprise a retrovirus into which a foreign gene that encodes a foreign protein is incorporated in place of normal retroviral RNA. When retroviral RNA enters a host cell coincident with infection, the foreign gene is also introduced into the cell, and may then be integrated into host cell DNA as if it were part of the retroviral genome. Expression of this foreign gene within the host results in expression of the foreign protein.

[0197] Most retroviral vector systems which have been developed for gene therapy are based on murine retroviruses. Such retroviruses exist in two forms, as free viral particles referred to as virions, or as proviruses integrated into host cell DNA. The virion form of the virus contains the structural and enzymatic proteins of the retrovirus (including the enzyme reverse transcriptase), two RNA copies of the viral genome, and portions of the source cell plasma membrane containing viral envelope glycoprotein. The retroviral genome is organized into four main regions: the Long Terminal Repeat (LTR), which contains cis-acting elements necessary for the initiation and termination of transcription and is situated both 5' and 3' of the coding genes, and the three coding genes gag, pol, and env. These three genes gag, pol, and env encode, respectively, internal viral structures, enzymatic proteins (such as integrase), and the envelope glycoprotein (designated gp70 and p15e) which confers infectivity and host range specificity of the virus, as well as the "R" peptide of undetermined function.

[0198] Separate packaging cell lines and vector producing cell lines have been developed because of safety concerns regarding the uses of retroviruses, including their use in expression constructs as provided by the present invention. Briefly, this methodology employs the use of two components, a retroviral vector and a packaging cell line (PCL). The retroviral vector contains long terminal repeats (LTRs), the foreign DNA to be transferred and a packaging sequence (y). This retroviral vector will not reproduce by itself because the genes which encode structural and envelope proteins are not included within the vector genome. The PCL contains genes encoding the gag, pol, and env proteins, but does not contain the packaging signal "y". Thus, a PCL can only form empty virion particles by itself. Within this general method, the retroviral vector is introduced into the PCL, thereby creating a vector-producing cell line (VCL). This VCL manufactures virion particles containing only the retroviral vector's (foreign) genome, and therefore has previously been considered to be a safe retrovirus vector for therapeutic use.

[0199] "Retroviral vector construct" refers to an assembly which is, within preferred embodiments of the invention, capable of directing the expression of a sequence(s) or gene(s) of interest, such as binding domain-immunoglobulin fusion encoding nucleic acid sequences. Briefly, the retroviral vector construct must include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR. A wide variety of heterologous sequences may be included within the vector construct, including for example, sequences which encode a protein (e.g., cytotoxic protein, disease-associated antigen, immune accessory molecule, or replacement gene), or which are useful as a molecule itself (e.g., as a ribozyme or antisense sequence).

[0200] Retroviral vector constructs of the present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses (see, e.g., RNA Tumor Viruses, Second Edition, Cold Spring

Harbor Laboratory, 1985). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; Rockville, Md.), or isolated from known sources using commonly available techniques. Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral vector constructs, packaging cells, or producer cells of the present invention given the disclosure provided herein, and standard recombinant techniques (e.g., Sambrook et al, *Molecular Cloning: A Laboratory Manual,* 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS* 82:488, 1985).

[0201] Suitable promoters for use in viral vectors generally may include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller, et al., *Biotechniques* 7:980-990 (1989), or any other promoter (e.g., cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, pol III, and β-actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein, and may be from among either regulated promoters or promoters as described above.

[0202] As described above, the retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, ψ-2, ψ-AM, PA12, T19-14X, VT-19-17-H2, ψCRE, ψCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, *Human Gene Therapy,* 1:5-14 (1990). The vector may transduce the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and $CaPO_4$ precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

[0203] The producer cell line generates infectious retroviral vector particles which include the nucleic acid sequence(s) encoding the binding domain-immunoglobulin fusion polypeptides or fusion proteins. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either in vitro or in vivo. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the binding domain-immunoglobulin fusion polypeptide or fusion protein. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, circulating peripheral blood mononuclear and polymorphonuclear cells including myelomonocytic cells, lymphocytes, myoblasts, tissue macrophages, dendritic cells, Kupffer cells, lymphoid and reticuloendothelia cells of the lymph nodes and spleen, keratinocytes, endothelial cells, and bronchial epithelial cells.

[0204] As another example of an embodiment of the invention in which a viral vector is used to prepare the recombinant binding domain-immunoglobulin fusion expression construct, in one preferred embodiment, host cells transduced by a recombinant viral construct directing the expression of binding domain-immunoglobulin fusion polypeptides or fusion proteins may produce viral particles containing expressed binding domain-immunoglobulin fusion polypeptides or fusion proteins that are derived from portions of a host cell membrane incorporated by the viral particles during viral budding.

[0205] In another aspect, the present invention relates to host cells containing the above described recombinant binding domain-immunoglobulin fusion expression constructs. Host cells are genetically engineered (transduced, transformed or transfected) with the vectors and/or expression constructs of this invention which may be, for example, a cloning vector, a shuttle vector or an expression construct. The vector or construct may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying particular genes such as genes encoding binding domain-immunoglobulin fusion polypeptides or binding domain-immunoglobulin fusion fusion proteins. The culture conditions for particular host cells selected for expression, such as temperature, pH and the like, will be readily apparent to the ordinarily skilled artisan.

[0206] The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Representative examples of appropriate host cells according to the present invention include, but need not be limited to, bacterial cells, such as *E. coli*, Streptomyces, *Salmonella typhimurium;* fungal cells, such as yeast; insect cells, such as Drosophila S2 and Spodoptera Sf9; animal cells, such as CHO, COS or 293 cells; adenoviruses; plant cells, or any suitable cell already adapted to in vitro propagation or so established de novo. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

[0207] Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, *Cell* 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences, for example as described herein regarding the preparation of binding domain-immunoglobulin fusion expression constructs. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements. Introduction of the construct into the host cell can be effected by a variety of methods with which those skilled in the art will be familiar, including but not limited to, for example, calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis et al., 1986 *Basic Methods in Molecular Biology*).

[0208] The present invention binding domain-immunoglobulin fusion proteins, or compositions comprising one or more polynucleotides encoding same as described herein, (for example, to be administered under conditions and for a time sufficient to permit expression of a binding domain-immunoglobulin fusion protein in a host cell in vivo or in

vitro), may be formulated into pharmaceutical compositions for administration according to well known methodologies. Pharmaceutical compositions generally comprise one or more recombinant expression constructs, and/or expression products of such constructs, in combination with a pharmaceutically acceptable carrier, excipient or diluent. Such carriers will be nontoxic to recipients at the dosages and concentrations employed. For nucleic acid-based formulations, or for formulations comprising expression products of the subject invention recombinant constructs, about 0.01 μg/kg to about 100 mg/kg body weight will be adminstered, typically by the intradermal, subcutaneous, intramuscular or intravenous route, or by other routes. A preferred dosage is about 1 μg/kg to about 1 mg/kg, with about 5 μg/kg to about 200 μg/kg particularly preferred. It will be evident to those skilled in the art that the number and frequency of administration will be dependent upon the response of the host. "Pharmaceutically acceptable carriers" for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remingtons Pharmaceutical Sciences,* Mack Publishing Co. (A. R. Gennaro edit. 1985). For example, sterile saline and phosphate-buffered saline at physiological pH may be used. Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. Id. at 1449. In addition, antioxidants and suspending agents may be used. Id.

[0209] "Pharmaceutically acceptable salt" refers to salts of the compounds of the present invention derived from the combination of such compounds and an organic or inorganic acid (acid addition salts) or an organic or inorganic base (base addition salts). The compounds of the present invention may be used in either the free base or salt forms, with both forms being considered as being within the scope of the present invention.

[0210] The pharmaceutical compositions that contain one or more binding domain-immunoglobulin fusion protein encoding constructs (or their expressed products) may be in any form which allows for the composition to be administered to a patient. For example, the composition may be in the form of a solid, liquid or gas (aerosol). Typical routes of administration include, without limitation, oral, topical, parenteral (e.g., sublingually or buccally), sublingual, rectal, vaginal, and intranasal. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal, intracavernous, intrathecal, intrameatal, intraurethral injection or infusion techniques. The pharmaceutical composition is formulated so as to allow the active ingredients contained therein to be bioavailable upon administration of the composition to a patient. Compositions that will be administered to a patient take the form of one or more dosage units, where for example, a tablet may be a single dosage unit, and a container of one or more compounds of the invention in aerosol form may hold a plurality of dosage units.

[0211] For oral administration, an excipient and/or binder may be present. Examples are sucrose, kaolin, glycerin, starch dextrins, sodium alginate, carboxymethylcellulose and ethyl cellulose. Coloring and/or flavoring agents may be present. A coating shell may be employed.

[0212] The composition may be in the form of a liquid, e.g., an elixir, syrup, solution, emulsion or suspension. The liquid may be for oral administration or for delivery by injection, as two examples. When intended for oral administration, preferred compositions contain, in addition to one or more binding domain-immunoglobulin fusion construct or expressed product, one or more of a sweetening agent, preservatives, dye/colorant and flavor enhancer. In a composition intended to be administered by injection, one or more of a surfactant, preservative, wetting agent, dispersing agent, suspending agent, buffer, stabilizer and isotonic agent may be included.

[0213] A liquid pharmaceutical composition as used herein, whether in the form of a solution, suspension or other like form, may include one or more of the following adjuvants: sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or digylcerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Physiological saline is a preferred adjuvant. An injectable pharmaceutical composition is preferably sterile.

[0214] It may also be desirable to include other components in the preparation, such as delivery vehicles including but not limited to aluminum salts, water-in-oil emulsions, biodegradable oil vehicles, oil-in-water emulsions, biodegradable microcapsules, and liposomes. Examples of immunostimulatory substances (adjuvants) for use in such vehicles include N-acetylmuramyl-L-alanine-D-isoglutamine (MDP), lipopolysaccharides (LPS), glucan, IL-12, GM-CSF, gamma interferon and IL-15.

[0215] While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration and whether a sustained release is desired. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (e.g., polylactic galactide) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Pat. Nos. 4,897,268 and 5,075,109. In this regard, it is preferable that the microsphere be larger than approximately 25 microns.

[0216] Pharmaceutical compositions may also contain diluents such as buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with nonspecific serum albumin are exemplary appropriate diluents. Preferably,

product is formulated as a lyophilizate using appropriate excipient solutions (e.g., sucrose) as diluents.

[0217] As described above, the subject invention includes compositions capable of delivering nucleic acid molecules encoding binding domain-immunoglobulin fusion proteins. Such compositions include recombinant viral vectors (e.g., retroviruses (see WO 90/07936, WO 91/02805, WO 93/25234, WO 93/25698, and WO 94/03622), adenovirus (see Berkner, *Biotechniques* 6:616-627, 1988; Li et al., *Hum. Gene Ther.* 4:403-409, 1993; Vincent et al., *Nat. Genet.* 5:130-134, 1993; and Kolls et al., *Proc. Natl. Acad. Sci. USA* 91:215-219, 1994), pox virus (see U.S. Pat. No. 4,769,330; U.S. Pat. No. 5,017,487; and WO 89/01973)), recombinant expression construct nucleic acid molecules complexed to a polycationic molecule (see WO 93/03709), and nucleic acids associated with liposomes (see Wang et al., *Proc. Natl. Acad. Sci. USA* 84:7851, 1987). In certain embodiments, the DNA may be linked to killed or inactivated adenovirus (see Curiel et al., *Hum. Gene Ther.* 3:147-154, 1992; Cotton et al., *Proc. Natl. Acad. Sci. USA* 89:6094, 1992). Other suitable compositions include DNA-ligand (see Wu et al., *J. Biol. Chem.* 264:16985-16987, 1989) and lipid-DNA combinations (see Felgner et al., *Proc. Natl. Acad. Sci. USA* 84:7413-7417, 1989).

[0218] In addition to direct in vivo procedures, ex vivo procedures may be used in which cells are removed from a host, modified, and placed into the same or another host animal. It will be evident that one can utilize any of the compositions noted above for introduction of binding domain-immunoglobulin fusion proteins or of binding domain-immunoglobulin fusion protein encoding nucleic acid molecules into tissue cells in an ex vivo context. Protocols for viral, physical and chemical methods of uptake are well known in the art.

[0219] Accordingly, the present invention is useful for treating a patient having a B-cell disorder or a malignant condition, or for treating a cell culture derived from such a patient. As used herein, the term "patient" refers to any warm-blooded animal, preferably a human. A patient may be afflicted with cancer or a malignant condition, such as B-cell lymphoma, or may be normal (i.e., free of detectable disease and infection). A "cell culture" includes any preparation amenable to ex vivo treatment, for example a preparation containing immunocompetent cells or isolated cells of the immune system (including, but not limited to, T cells, macrophages, monocytes, B cells and dendritic cells). Such cells may be isolated by any of a variety of techniques well known to those of ordinary skill in the art (e.g., Ficoll-hypaque density centrifugation). The cells may (but need not) have been isolated from a patient afflicted with a B-cell disorder or a malignant condition, and may be reintroduced into a patient after treatment.

[0220] A liquid composition intended for either parenteral or oral administration should contain an amount of binding domain-immunoglobulin fusion protein encoding construct or expressed product such that a suitable dosage will be obtained. Typically, this amount is at least 0.01 wt % of a binding domain-immunoglobulin fusion construct or expressed product in the composition. When intended for oral administration, this amount may be varied to be between 0.1 and about 70% of the weight of the composition. Preferred oral compositions contain between about 4% and about 50% of binding domain-immunoglobulin fusion construct or expressed product(s). Preferred compositions and preparations are prepared so that a parenteral dosage unit contains between 0.01 to 1% by weight of active compound.

[0221] The pharmaceutical composition may be intended for topical administration, in which case the carrier may suitably comprise a solution, emulsion, ointment or gel base. The base, for example, may comprise one or more of the following: petrolatum, lanolin, polyethylene glycols, beeswax, mineral oil, diluents such as water and alcohol, and emulsifiers and stabilizers. Thickening agents may be present in a pharmaceutical composition for topical administration. If intended for transdermal administration, the composition may include a transdermal patch or iontophoresis device. Topical formulations may contain a concentration of the binding domain-immunoglobulin fusion construct or expressed product of from about 0.1 to about 10% w/v (weight per unit volume).

[0222] The composition may be intended for rectal administration, in the form, e.g., of a suppository which will melt in the rectum and release the drug. The composition for rectal administration may contain an oleaginous base as a suitable nonirritating excipient. Such bases include, without limitation, lanolin, cocoa butter and polyethylene glycol.

[0223] In the methods of the invention, the binding domain-immunoglobulin fusion encoding constructs or expressed product(s) may be administered through use of insert(s), bead(s), timed-release formulation(s), patch(es) or fast-release formulation(s).

[0224] The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1

[0225] Cloning of the 2H7 Variable Regions and Construction and Sequencing of 2H7scFV-IG

[0226] This Example illustrates the cloning of cDNA molecules that encode the heavy chain and light chain variable regions of the monoclonal antibody 2H7. This Example also demonstrates the construction, sequencing, and expression of 2H7scFv-Ig.

[0227] Hybridoma cells expressing 2H7 monoclonal antibody that specifically bound to CD20 were provided by Ed Clark at the University of Washington, Seattle, Wash. Prior to harvesting, hybridoma cells were kept in log phase growth for several days in RPMI 1640 media Invitrogen/Life Technologies, Gaithersburg, Md.) supplemented with glutamine, pyruvate, DMEM non-essential amino acids, and penicillin-streptomycin. Cells were pelleted by centrifugation from the culture medium, and 2×10^7 cells were used to prepare RNA. RNA was isolated from the 2H7-producing hybridoma cells using the Pharmingen (San Diego, Calif.) total RNA isolation kit (Catalog #45520K) according to the manufacturer's instructions accompanying the kit. One microgram (1 μg) of total RNA was used as template to prepare cDNA by reverse transcription. The RNA and 300 ng random primers were combined and denatured at 72° C. for 10 minutes prior to addition of enzyme. Superscript II reverse transcriptase (Life Technologies) was added to the RNA plus primer

mixture in a total volume of 25 μl in the presence of 5xsecond strand buffer and 0.1 M DTT provided with the enzyme. The reverse transcription reaction was allowed to proceed at 42° C. for one hour.

[0228] The 2H7 cDNA generated in the randomly primed reverse transcriptase reaction and V region specific primers were used to amplify by PCR the variable regions for the light and heavy chain of the 2H7 antibody. The V region specific primers were designed using the published sequence (Genbank accession numbers M17954 for V_L and M17953 for V_H) as a guide. The two variable chains were designed with compatible end sequences so that an scFv could be assembled by ligation of the two V regions after amplification and restriction enzyme digestion.

[0229] A $(gly_4ser)_3$ peptide linker to be inserted between the two V regions was incorporated by adding the extra nucleotides to the antisense primer for the V_L of 2H7. A Sac I restriction site was also introduced at the junction between the two V regions. The sense primer used to amplify the 2H7 V_L, that included a HindIII restriction site and the light chain leader peptide was 5'-gtc aag ctt gcc gcc atg gat ttt caa gtg cag att ttt cag c-3' (SEQ ID NO:______). The antisense primer was 5'-gtc gtc gag ctc cca cct cct cca gat cca cca ccg ccc gag cca ccg cca cct ttc agc tcc agc ttg gtc cc-3' (SEQ ID NO:______). The reading frame of the V region is indicated as a bold, underlined codon. The Hind III and SacI sites are indicated by underlined italicized sequences.

[0230] The V_H domain was amplified without a leader peptide, but included a 5' SacI restriction site for fusion to the V_L and a BclI restriction site at the 3' end for fusion to various tails, including the human IgG1 Fc domain and the truncated forms of CD40 ligand, CD154. The sense primer was 5'-gct gct gag ctc tca ggc tta tct aca gca agt ctg g-3' (SEQ ID NO:______). The SacI site is indicated in italicized and underlined font, and the reading frame of the codon for the first amino acid of the V_H domain is indicated in bold, underlined type. The antisense primer was 5'-gtt gtc tga tca gag acg gtg acc gtg gtc cc-3' (SEQ ID NO:______). The BclI site is indicated in italicized, underlined type, and the last serine of the V_H domain sequence is indicated in bold, underlined type.

[0231] The scFv-Ig was assembled by inserting the 2H7 scFv HindIII-BclI fragment into pUC19 containing the human IgG1 hinge, CH2, and CH3 regions, which was digested with restriction enzymes, HindIII and BclI. After ligation, the ligation products were transformed into DH5α bacteria. Positive clones were screened for the properly inserted fragments using the SacI site at the V_L-V_H junction of 2H7 as a diagnostic site. The 2H7scFv-Ig cDNA was subjected to cycle sequencing on a PE 9700 thermocycler using a 25-cycle program by denaturing at 96° C. for 10 seconds, annealing at 50° C. for 30 seconds, and extending at 72° C. for 4 minutes. The sequencing primers were pUC forward and reverse primers and an internal primer that annealed to the CH2 domain human in the IgG constant region portion. Sequencing reactions were performed using the Big Dye Terminator Ready Sequencing Mix (PE-Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. Samples were subsequently purified using Centrisep columns (Catalog #CS-901, Princeton Separations, Adelphia, N.J.), the eluates dried in a Savant vacuum dryer, denatured in Template Suppression Reagent (PE-ABI), and analyzed on an ABI 310 Genetic Analyzer (PE-Applied Biosystems). The sequence was edited, translated, and analyzed using Vector Nti version 6.0 (Informax, North Bethesda, Md.). **FIG. 1** shows the cDNA and predicted amino acid sequence of the 2H7scFv-Ig construct.

Example 2

[0232] Expression of 2H7 scFv-Ig in Stable CHO Cell Lines

[0233] This Example illustrates expression of 2H7scFv-Ig in a eukaryotic cell line and characterization of the expressed 2H7scFv-Ig by SDS-PAGE and by functional assays, including ADCC and complement fixation.

[0234] The 2H7scFv-Ig HindIII-XbaI (~1.6 kb) fragment with correct sequence was inserted into the mammalian expression vector pD18, and DNA from positive clones was amplified using QIAGEN plasmid preparation kits (QIAGEN, Valencia, Calif.). The recombinant plasmid DNA (100 μg) was then linearized in a nonessential region by digestion with AscI, purified by phenol extraction, and resuspended in tissue culture media, Excell 302 (Catalog #14312-79P, JRH Biosciences, Lenexa, Kans.). Cells for transfection, CHO DG44 cells, were kept in logarithmic growth, and 10^7 cells harvested for each transfection reaction. Linearized DNA was added to the CHO cells in a total volume of 0.8 ml for electroporation.

[0235] Stable production of the 2H7 scFv-Ig fusion protein (SEQ. ID NO:10) was achieved by electroporation of a selectable, amplifiable plasmid, pD18, containing the 2H7 scFv-Ig cDNA under the control of the CMV promoter, into Chinese Hamster Ovary (CHO) cells (all cell lines from American Type Culture Collection, Manassas, Va., unless otherwise noted). The 2H7 expression cassette was subcloned downstream of the CMV promoter into the vector multiple cloning site as a ~1.6 kb HindIII-XbaI fragment. The pD18 vector is a modified version of pcDNA3 encoding the DHFR selectable marker with an attenuated promoter to increase selection pressure for the plasmid. Plasmid DNA was prepared using Qiagen maxiprep kits, and purified plasmid was linearized at a unique AscI site prior to phenol extraction and ethanol precipitation. Salmon sperm DNA (Sigma-Aldrich, St. Louis, Mo.) was added as carrier DNA, and 100 μg each of plasmid and carrier DNA was used to transfect 10^7 CHO DG44 cells by electroporation. Cells were grown to logarithmic phase in Excell 302 media (JRH Biosciences) containing glutamine (4 mM), pyruvate, recombinant insulin, penicillin-streptomycin, and 2xDMEM nonessential amino acids (all from Life Technologies, Gaithersburg, Md.), hereafter referred to as "Excell 302 complete" media. Media for untransfected cells also contained HT (diluted from a 100xsolution of hypoxanthine and thymidine) (Invitrogen/Life Technologies). Media for transfections under selection contained varying levels of methotrexate (Sigma-Aldrich) as selective agent, ranging from 50 nM to 5 μM. Electroporations were performed at 275 volts, 950 μF. Transfected cells were allowed to recover overnight in non-selective media prior to selective plating in 96 well flat bottom plates (Costar) at varying serial dilutions ranging from 125 cells/well to 2000 cells/well. Culture media for cell cloning was Excell 302 complete, containing 100 nM methotrexate. Once clonal outgrowth was sufficient, serial dilutions of culture supernatants from master wells were

screened for binding to CD20-CHO transfected cells. The clones with the highest production of the fusion protein were expanded into T25 and then T75 flasks to provide adequate numbers of cells for freezing and for scaling up production of the 2H7scFvIg. Production levels were further increased in cultures from three clones by progressive amplification in methotrexate containing culture media. At each successive passage of cells, the Excell 302 complete media contained an increased concentration of methotrexate, such that only the cells that amplified the DHFR plasmid could survive.

[0236] Supernatants were collected from CHO cells expressing the 2H7scFv-Ig, filtered through 0.2 μm PES express filters (Nalgene, Rochester, N.Y.) and were passed over a Protein A-agarose (IPA 300 crosslinked agarose) column (Repligen, Needham, Mass.). The column was washed with PBS, and then bound protein was eluted using 0.1 M citrate buffer, pH 3.0. Fractions were collected and eluted protein was neutralized using 1M Tris, pH 8.0, prior to dialysis overnight in PBS. Concentration of the purified 2H7scFv-Ig (SEQ ID NO:______) was determined by absorption at 280 nm. An extinction coefficient of 1.77 was determined using the protein analysis tools in the Vector Nti Version 6.0 Software package (Informax, North Bethesda, Md.). This program uses the amino acid composition data to calculate extinction coefficients.

[0237] Production levels of 2H7scFv-Ig by transfected, stable CHO cells were analyzed by flow cytometry. Purified 2H7scFv-Ig to CHO cells was allowed to bind to CHO cells that expressed CD20 (CD20 CHO) and analyzed by flow cytometry using a fluorescein-conjugated anti-human IgG second step reagent (Catalog Numbers H10101 and H10501, CalTag, Burlingame, Calif.). **FIG. 2** (top) shows a standard curve generated by titration of 2H7scFv-Ig binding to CD20 CHO. At each concentration of 2H7scFv-Ig, the mean brightness of the fluorescein signal in linear units is shown. Supernatants collected from T flasks containing stable CHO cell clones expressing 2H7scFv-Ig were then allowed to bind to CD20 CHO and the binding was analyzed by flow cytometry. The fluorescein signal generated by 2H7scFv-Ig contained in the supernatants was measured and the 2H7scFv-Ig concentration in the supernatants was calculated from the standard curve (**FIG. 2**, bottom).

[0238] Purified 2H7scFv-Ig (SEQ ID NO:______) was analyzed by electrophoresis on SDS-Polyacrylamide gels. Samples of 2H7scFv-Ig, purified by independent Protein A Agarose column runs, were boiled in SDS sample buffer without reduction of disulfide bonds and applied to SDS 10% Tris-BIS gels (Catalog #NP0301, Novex, Carlsbad, Calif.). Twenty micrograms of each purified batch was loaded on the gels. The proteins were visualized after electrophoresis by Coomassie Blue staining (Pierce Gel Code Blue Stain Reagent, Catalog #24590, Pierce, Rockford, Ill.), and destaining in distilled water. Molecular weight markers were included on the same gel (Kaleidoscope Prestained Standards, Catalog #161-0324, Bio-Rad, Hercules, Calif.). The results are presented in **FIG. 3**. The numbers above the lanes designate independent purification batches. The molecular weights in kilodaltons of the size markers are indicated on the left side of the figure. Further experiments with alternative sample preparation conditions indicated that reduction of disulfide bonds by boiling the protein in SDS sample buffer containing DTT or 2-mercaptoethanol caused the 2H7scFv-Ig to aggregate.

[0239] Any number of other immunological parameters may be monitored using routine assays that are well known in the art. These may include, for example, antibody dependent cell-mediated cytotoxicity (ADCC) assays, secondary in vitro antibody responses, flow immunocytofluorimetric analysis of various peripheral blood or lymphoid mononuclear cell subpopulations using well established marker antigen systems, immunohistochemistry or other relevant assays. These and other assays may be found, for example, in Rose et al. (Eds.), *Manual of Clinical Laboratory Immunology*, 5th Ed., 1997 American Society of Microbiology, Washington, D.C.

[0240] The ability of 2H7scFv-Ig to kill CD20 positive cells in the presence of complement was tested using B cell lines Ramos and Bjab. Rabbit complement (Pel-Freez, Rogers, A K) was used in the assay at a final dilution of 1/10. Purified 2H7scFv-Ig was incubated with B cells and complement for 45 minutes at 37° C., followed by counting of live and dead cells by trypan blue exclusion. The results in **FIG. 4A** show that in the presence of rabbit complement, 2H7scFv-Ig lysed B cells expressing CD20.

[0241] The ability of 2H7scFv-Ig to kill CD20 positive cells in the presence of peripheral blood mononuclear cells (PBMC) was tested by measuring the release of ^{51}Cr from labeled Bjab cells in a 4-hour assay using a 100:1 ratio of PBMC to Bjab cells. The results shown in **FIG. 4B** indicated that 2H7scFv-Ig can mediate antibody dependent cellular cytotoxicity (ADCC) because the release of ^{51}Cr was higher in the presence of both PBMC and 2H7scFv-Ig than in the presence of either PBMC or 2H7scFv-Ig alone.

Example 3

[0242] Effect of Simultaneous Ligation of CD20 and CD40 on Growth of Normal B Cells, and on CD95 Expression, and Induction of Apoptosis

[0243] This example illustrates the effect of cross-linking of CD20 and CD40 expressed on the cell surface on cell proliferation.

[0244] Dense resting B cells were isolated from human tonsil by a Percoll step gradient and T cells were removed by E-rosetting. Proliferation of resting, dense tonsillar B cells was measured by uptake of ^{3}[H]-thymidine during the last 12 hours of a 4-day experiment. Proliferation was measured in quadruplicate cultures with means and standard deviations as shown. Murine anti-human CD20 mAb 1F5 (anti-CD20) was used alone or was cross-linked with anti-murine κ mAb 187.1 (anti-CD20XL). CD40 activation was accomplished using soluble human CD154 fused with murine CD8 (CD154) (Hollenbaugh et al., *EMBO J.* 11: 4212-21 (1992)), and CD40 cross-linking was accomplished using anti-murine CD8 mAb 53-6 (CD154XL). This procedure allowed simultaneous cross-linking of CD20 and CD40 on the cell surface. The results are presented in **FIG. 5**.

[0245] The effect of CD20 and CD40 cross-linking on Ramos cells, a B lymphoma cell line, was examined. Ramos cells were analyzed for CD95 (Fas) expression and percent apoptosis eighteen hours after treatment (no goat anti-mouse IgG (GAM)) and/or cross-linking (+GAM) using murine mAbs that specifically bind CD20 (1F5) and CD40 (G28-5). Control cells were treated with a non-binding isotype control (64.1) specific for CD3.

[0246] Treated Ramos cells were harvested, incubated with FITC-anti-CD95, and analyzed by flow cytometry to determine the relative expression level of Fas on the cell surface after CD20 or CD40 cross-linking. Data is plotted as mean fluorescence of cells after treatment with the stimuli indicated (**FIG. 6A**).

[0247] Treated Ramos cells from the same experiment were harvested and binding of annexin V was measured to indicate the percentage apoptosis in the treated cultures. Apoptosis was measured by binding of Annexin V 18 hours after cross-linking of CD20 and CD40 using 1F5 and G28-5 followed by cross-linking with GAM. Binding of Annexin V was measured using a FITC-Annexin V kit (Catalog #PN-IM2376, Immunotech, Marseille, France,). Annexin V binding is known to be an early event in progression of cells into apoptosis. Apoptosis, or programmed cell death, is a process characterized by a cascade of catabolic reactions leading to cell death by suicide. In the early phase of apoptosis, before cells change morphology and hydrolyze DNA, the integrity of the cell membrane is maintained but cells lose the asymmetry of their membrane phospholipids, exposing negatively charged phospholipids, such as phosphatidylserine, at the cell surface. Annexin V, a calcium and phopholipid binding protein, binds preferentially and with high affinity to phosphatidylserine. Results demonstrating the effect of cross-linking both CD20 and CD40 on expression of the FAS receptor (CD95) are presented in **FIG. 6B**. The effect of cross-linking of both CD20 and CD40 on Annexin V binding to cells is shown in **FIG. 6B**.

Example 4

[0248] Construction and Characterization of 2H7 scFv-CD154 Fusion Proteins

[0249] To construct a molecule capable of binding to both CD20 and CD40, cDNA encoding the 2H7 scFv was fused with cDNA encoding CD154, the CD40 ligand. The 2H7 scFv cDNA encoded on the HindIII-BclI fragment was removed from the 2H7 scFvIg construct, and inserted into a pD18 vector along with a BamHI-XbaI cDNA fragment encoding the extracellular domain of human CD154. The extracellular domain is encoded at the carboxy terminus of CD154, similar to other type II membrane proteins.

[0250] The extracellular domain of human CD154 was PCR amplified using cDNA generated with random primers and RNA from human T lymphocytes activated with PHA (phytohemagglutinin). The primer sets included two different 5' or sense primers that created fusion junctions at two different positions within the extracellular domain of CD154. Two different fusion junctions were designed that resulted in a short or truncated form (form S4) including amino acids 108 (Glu)-261 (Leu)+(Glu), and a long or complete form (form L2) including amino acids 48 (Arg) -261 (Leu)+(Glu), of the extracellular domain of CD154, both constructed as BamHI-XbaI fragments. The sense primer which fuses the two different truncated extracellular domains to the 2H7scFv includes a BamHI site for cloning. The sense primer for the S4 form of the CD154 cDNA is designated SEQUENCE ID NO: 11 or CD154BAM108 and encodes a 34 mer with the following sequence: 5'-gtt gtc gga tcc aga aaa cag ctt tga aat gca a-3', while the antisense primer is designated SEQUENCE ID NO: 12 or CD154XBA and encodes a 44 mer with the following sequence: 5'-gtt gtt tct aga tta tca ctc gag ttt gag taa gcc aaa gga cg-3'.

[0251] The oligonucleotide primers used in amplifying the long form (L2) of the CD154 extracellular domain encoding amino acids 48 (Arg)-261 (Leu)+(Glu), were as follows: The sense primer designated CD154 BAM48 (SEQUENCE ID NO:13) encoded a 35-mer with the following sequence: 5'-gtt gtc gga tcc aag aag gtt gga caa gat aga ag-3'. The antisense primer designated or CD154XBA (SEQUENCE ID NO:______) encoded the 44-mer: 5'-gtt gtt tct aga tta tca ctc gag ttt gag taa gcc aaa gga cg-3'. Other PCR reaction conditions were identical to those used for amplifying the 2H7 scFv (see Example 1). PCR fragments were purified by PCR quick kits (QIAGEN, San Diego, Calif.), eluted in 30 μl ddH$_2$O, and digested with BamHI and XbaI (Roche) restriction endonucleases in a 40 μl reaction volume at 37° C. for 3 hours. Fragments were gel purified, purified using QIAEX kits according to the manufacturer's instructions (QIAGEN), and ligated along with the 2H7 HindIII-BclI fragment into the pD18 expression vector digested with HindIII+XbaI. Ligation reactions were transformed into DH5-alpha chemically competent bacteria and plated onto LB plates containing 100 μg/ml ampicillin. Transformants were grown overnight at 37° C., and isolated colonies used to inoculate 3 ml liquid cultures in Luria Broth containing 100 μg/ml ampicillin. Clones were screened after mini-plasmid preparations (QIAGEN) for insertion of both the 2H7 scFv and the CD154 extracellular domain fragments.

[0252] The 2H7scFv-CD154 construct cDNAs were subjected to cycle sequencing on a PE 9700 thermocycler using a 25-cycle program that included denaturating at 96° C., 10 seconds, annealing at 50° C. for 5 seconds, and extension at 60° C, for 4 minutes. The sequencing primers used were pD18 forward (SEQ ID NO:______: 5'-gtctatataagcagagctctggc-3') and pD18 reverse (SEQ ID NO:______: 5'-cgaggctgatcagcgagctctagca-3') primers. In addition, an internal primer was used that had homology to the human CD154 sequence (SEQ ID NO:______: 5'-ccgcaatttgaggattctgatcacc-3'). Sequencing reactions included primers at 3.2 pmol, approximately 200 ng DNA template, and 8 μl sequencing mix. Sequencing reactions were performed using the Big Dye Terminator Ready Sequencing Mix (PE-Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. Samples were subsequently purified using Centrisep columns (Princeton Separations, Adelphia, N.J.). The eluates were dried in a Savant speed-vacuum dryer, denatured in 20 μl template Suppression Reagent (ABI) at 95° C. for 2 minutes, and analyzed on an ABI 310 Genetic Analyzer (PE-Applied Biosystems). The sequence was edited, translated, and analyzed using Vector Nti version 6.0 (Informax, North Bethesda, Md.). The 2H7scFv-CD154 L2 cDNA sequence and predicted amino acid sequence is presented in **FIG. 7A**, and 2H7scFv-CD154 S4 cDNA sequence and predicted amino acid sequence is presented in **FIG. 7B**.

[0253] The binding activity of the 2H7 scFv-CD154 fusion proteins (SEQ. ID NO:______ and ______) to CD20 and CD40 simultaneously was determined by flow cytometry. The assay used CHO cell targets that express CD20. After a 45-minute incubation of CD20 CHO cells with supernatants from cells transfected with the 2H7 scFv-CD154 expression plasmid, the CD20 CHO cells were washed twice and incubated with biotin-conjugated CD40-Ig fusion protein in PBS/2% FBS. After 45 min, cells were washed twice and incubated with phycoerythrin (PE)-labeled strepavidin at 1:100 in PBS/2% FBS (Molecular

Probes, Eugene Oreg.). After an additional 30 min incubation, cells were washed 2× and were analyzed by flow cytometry. The results show that the 2H7 scFv-CD154 molecule was able to bind to CD20 on the cell surface and to capture biotin-conjugated CD40 from solution (**FIG. 8**).

[0254] To determine the effect of the 2H7scFv-CD154 on growth and viability of B lymphoma and lymphoblastoid cell lines, cells were incubated with 2H7scFv-CD154 L2 (SEQ. ID NO:______) for 12 hours and then examined for binding of Annexin V. Binding of Annexin V was measured using a FITC-Annexin V kit (Immunotech, Marseille, France, Catalog #PN-IM2376). B cell lines were incubated in 1 ml cultures with dilutions of concentrated, dialyzed supernatants from cells expressing secreted forms of the 2H7scFv-CD154 fusion proteins. The results are presented in **FIG. 9**.

[0255] The growth rate of the Ramos B lymphoma cell line in the presence of 2H7scFv-CD154 was examined by uptake of ^{3}H-thymidine for the last 6 hours of a 24-hour culture. The effect of 2H7scFv-CD154 on cell proliferation is shown in **FIG. 10**.

Example 5

[0256] Construction and Characterization of CytoxB Antibody Derivatives

[0257] CytoxB antibodies were derived from the 2H7 scFv-IgG polypeptide. The 2H7 scFv (see Example 1) was linked to the human IgG1 Fc domain via an altered hinge domain (see **FIG. 11**). Cysteine residues in the hinge region were substituted with serine residues by site-directed mutagenesis and other methods known in the art. The mutant hinge was fused either to a wild-type Fc domain to create one construct, designated CytoxB-MHWTG1C, or was fused to a mutated Fc domain (CytoxB-MHMG1C) that had additional mutations introduced into the CH2 domain. Amino acid residues in CH2 that are implicated in effector function are illustrated in **FIG. 11**. Mutations of one or more of these residues may reduce FcR binding and mediation of effector functions. In this example, the leucine residue 234 known in the art to be important to Fc receptor binding, was mutated in the 2H7 scFv fusion protein, CytoxB-[MG1H/MG1C]. In another construct, the human IgG1 hinge region was substituted with a portion of the human IgA hinge, which was fused to wild-type human Fc domain (CytoxB-IgAHWTHG1C). (See **FIG. 11**). This mutated binge region allows expression of a mixture of monomeric and dimeric molecules that retain functional properties of the human IgG1 CH2 and CH3 domains. Synthetic, recombinant cDNA expression cassettes for these molecules were constructed and polypeptides were expressed in CHODG44 cells according to methods described in Example 2.

[0258] Purified fusion protein derivatives of CytoxB-scFvIg molecules were analyzed by SDS-PAGE according to the methods described in Example 2. Polyacrylamide gels were run under non-reducing and reducing conditions. Two different molecule weight marker sets, BioRad prestained markers, (BioRad, Hercules, Calif.) and Novex Multimark molecular weight markers were loaded onto each gel. The migration patterns of the different constructs and of Rituximab™ are presented in **FIG. 12**.

[0259] The ability of the different derivatives of CytoxB-scFvIg molecules to mediate ADCC was measured using the Bjab B lymphoma cells as the target and freshly prepared human PBMCs as effector cells. (See Example 2). Effector to target ratios were varied as follows: 70:1, 35:1, and 18:1, with the number of Bjab cells per well remaining constant but the number of PBMCs were varied. Bjab cells were labeled for 2 hours with ^{51}Cr and aliquoted at a cell density of 5×10^{4} cells/well to each well of flat-bottom 96 well plates. Purified fusion proteins or rituximab were added at a concentration of 10 μg/ml to the various dilutions of PBMCs. Spontaneous release was measured without addition of PBMC or fusion protein, and maximal release was measured by the addition of detergent (1% NP-40) to the appropriate wells. Reactions were incubated for 4 hours, and 100 μl of culture supernatant was harvested to a Lumaplate (Packard Instruments) and allowed to dry overnight prior to counting cpm released. The results are presented in **FIG. 13**.

[0260] Complement dependent cytotoxicity (CDC) activity of the CytoxB derivatives was also measured. Reactions were performed essentially as described in Example 2. The results are presented in **FIG. 14** as percent of dead cells to total cells for each concentration of fusion protein.

Example 6

[0261] In vivo Studies in Macaques

[0262] Initial in vivo studies with CytoxB derivatives have been performed in nonhuman primates. **FIG. 15** shows data characterizing the serum half-life of CytoxB in monkeys. Measurements were performed on serum samples obtained from two different macaques (J99231 and K99334) after doses of 6 mg/kg were administered to each monkey on the days indicated by arrows. For each sample, the level of 2H7scFvIg present was estimated by comparison to a standard curve generated by binding of purified CytoxB-(MHWTG1C)-Ig fusion protein to CD20 CHO cells (see Example 2). The data are tabulated in the bottom panel of the **FIG. 15**.

[0263] The effect of CytoxB-(MHWTG1C)Ig fusion protein on levels of circulating CD40+ cells in macaques was investigated. Complete blood counts were performed at each of the days indicated in **FIG. 16**. In addition, FACS (fluorescence activated cell sorter) assays were performed on peripheral blood lymphocytes using a CD40-specific fluorescein conjugated antibody to detect B cells among the cell population. The percentage of positive cells was then used to calculate the number of B cells in the original samples. The data are graphed as thousands of B cells per microliter of blood measured at the days indicated after injection (**FIG. 16**).

Example 7

[0264] Construction and Expression of an anti-CD19 scFv-Ig Fusion Protein

[0265] An anti-CD19 scFv-Ig fusion protein was constructed, transfected into eukaryotic cells, and expressed according to methods presented in Examples 1, 2, and 5 and standard in the art. The variable heavy chain regions and variable light chain regions were cloned from RNA isolated from hybridoma cells producing antibody HD37, which specifically binds to CD19. Expression levels of a HD37scFv-IgAHWTG1C and a HD37scFv-IgMHWTG1C were measured and compared to a standard curve generated using purified HD37 scFvIg. The results are presented in **FIG. 17**.

Example 8

[0266] Construction and Expression of an Anti-L6 scFv-Ig Fusion Protein

[0267] An scFv-Ig fusion protein was constructed using variable regions derived from an anti-carcinoma mAb, L6. The fusion protein was constructed, transfected into eukaryotic cells, and expressed according to methods presented in Examples 1, 2, and 5 and standard in the art. Expression levels of L6scFv-IgAHWTG1C and L6scFv-IgMHWTG1C were measured and compared to a standard curve generated using purified HD37 scFvIg. The results are presented in **FIG. 18**.

Example 9

[0268] Characterization of Various scFv-Ig Fusion Proteins

[0269] In addition to the scFv-Ig fusion protein already described, G28-1 (anti-CD37) scFv-Ig fusion proteins were prepared essentially as described in Examples 1 and 5. The variable regions of the heavy and light chains were cloned according to methods known in the art. ADCC activity of malian vector constructs containing an anti-CD20 single chain Fv, 2H7 scFv. Total RNA was isolated from peripheral blood mononuclear cells (PBMC) from llama blood (Triple J Farms, Bellingham, Wash.) by lysing the lymphocytes in TRIzol® (Invitrogen Life Technologies, Carlsbad, Calif.) according to the manufacturer's instructions. One microgram (1 μg) of total RNA was used as template to prepare cDNA by reverse transcription. The RNA and 200 ng random primers were combined and denatured at 72° C. for 10 minutes prior to addition of enzyme. Superscript II reverse transcriptase (Invitrogen Life Technologies) was added to the RNA plus primer mixture in a total volume of 25 μl in the presence of 5xsecond strand buffer and 0.1 M DTT provided with the enzyme. The reverse transcription reaction was allowed to proceed at 42° C. for one hour. The cDNA was amplified by PCR using sequence specific primers. The 5' primers were designed according to published sequences for the V_{HH} and V_H domains of camelids. The 3' primer, which was used to amplify all three isotypes, was designed using mammalian CH3 domain sequences as a guide. The following specific primers were used. The Bcl and XbaI sites are indicated by underlined italicized sequences.

```
5' primer for llama IgGI constant region
LLG1-5'bgl: 5'-gtt gttgatcaa gaa cca cat gga gga tgc acg tg-3'            (SEQ ID NO:__)

5' primer for llama IgG2 constant region
LLG2-5'bgl: 5'-gtt gttgatcaa gaa ccc aag aca cca aaa cc-3'                 (SEQ ID NO:__)

5' primer for llama IgG3 constant region
LLG3-5'bgl: 5'-gtt gttgatcaa gcg cac cac agc gaa gac ccc-3'                (SEQ ID NO:  x)

3' primer for llama IgGi, IgG2, and IgG3 constant regions
LLG123-3'X: 5'-gtt gtt tct aga tta cta ttt acc cga aga ctg ggt gat gga-3'  (SEQ ID NO:  )
```

2H7-MHWTG1C, 2H7-IgAHWTG1C, G28-1-MHWTG1C, G28-1 IgAHWTG1C, HD37-MHWTG1C, and HD37-IgAHWTG1C was determined according to methods described above (see Example 2). Results are presented in **FIG. 19**. ADCC activity of L6scFv-IgAHWTG1C and L6scFv-IgMHWTG1C was measured using the 2981 human lung carcinoma cell line. The results are presented in **FIG. 20**. The murine L6 monoclonal antibody is known not to exhibit ADCC activity.

[0270] The purified proteins were analyzed by SDS-PAGE under reducing and non-reducing conditions. Samples were prepared and gels run essentially as described in Examples 2 and 5. The results for the L6 and 2H7 scFv-Ig fusion proteins are presented in **FIG. 21** and the results for the G28-1 and HD37 scFv-Ig fusion proteins are presented in **FIG. 22**.

Example 10

[0271] Construction and Expression of Anti-CD20 scFv-llama Ig Fusion Proteins

[0272] This Example illustrates the cloning of llama IgG1, IgG2, and IgG3 constant region domains and the construction of immunoglobulin fusion proteins with each of the three constant regions and anti-CD20 scFv.

[0273] The constant regions of llama IgG1, IgG2, and IgG3 immunoglobulins were cloned and inserted into mam-

[0274] PCR fragments of the expected size were cloned into TOPO® cloning vectors (Invitrogen Life Technologies) and then were sequenced. The sense sequencing primer, LLseqsense, had the sequence 5'-ctg aga tcg agt tca gct g-3' (SEQ ID NO:______), and the antisense primer, LLseqAS, had the sequence 5'-cct cct ttg gct ttg tct c-3' (SEQ ID NO:______. Sequencing was performed as described in Example 1. **FIG. 23** compares the amino acid sequence of the three isotype llama constant regions containing the hinge, CH2, and CH3 domains with the amino acid sequence of human IgG1 hinge, CH2, and CH3 domains.

[0275] After verifying the sequence, the amplified PCR products were digested with restriction enzymes BclI and XbaI to create compatible restriction sites. The digested fragments were then gel-purified, and the DNA was eluted using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, Calif.). The 2H7scFv-Ig pD18 mammalian expression vector construct (see Example 2) was digested with BclI and XbaI to remove the human IgG hinge, CH2, and CH3 domains. The pD18 vector is a modified derivative of pcDNA3 that contains an attenuated DHFR gene, which serves as a selectable marker for mammalian expression (Hayden et al., *Tissue Antigens* 48:242-54 (1996)). The purified llama IgG1, IgG2, and IgG3 constant region PCR products were ligated by T4 DNA ligase (Roche Molecular Biochemicals, Indianapolis, Ind.) into the double-digested 2H7 scFv-pD18 vector at room temperature overnight according to the manufacturer's instructions. After ligation,

the ligation products were transformed into *E. coli* DH5α bacteria (BD Biosciences, Palo Alto, Calif.) and plated according to standard molecular biology procedures and manufacturer's instructions. Isolated colonies were chosen to screen for transformants containing the correct inserts.

[0276] For expression of the encoded polypeptides, plasmid DNA from positive clones was transiently transfected into COS-7 cells using DEAE-dextran (Hayden et al., *Ther Immunol.* 1:3-15 (1994)). COS-7 cells were seeded at approximately 3×10^6 cells per 150 mm plate and grown overnight until the cells were about 75% confluent. Cells were then washed once with serum-free DMEM (Invitrogen Life Technologies, Grand Island, N.Y.). Transfection supernatant (10 ml) containing 400 μg/ml DEAE-dextran, 0.1 mM chloroquine, and 5 μg/ml of the DNA constructs were added to the cells, which were then incubated at 37° C. for 3-4 hrs. After incubation, cells were pulsed with 10 ml of 10% dimethyl sulfoxide (DMSO) in 1×PBS at room temperature for 2 minutes. Cells were then placed back into fully supplemented DMEM/10% FBS (1% L-glutamine, 1% penicillin/streptomycin, 1% sodium pyruvate, 1% MEM essential amino acids) (Invitrogen Life Technologies). After 24 hours, the media was replaced with serum-free fully supplemented DMEM (Invitrogen Life Technologies), and the cells were maintained up to 21 days with media changes every 3-4 days.

[0277] Ig-fusion proteins were purified by passing COS cell culture supernatants through Protein A Agarose (Repligen, Cambridge, Mass.) columns. After application of the culture supernatant, the Protein A columns were then washed with 1×PBS (Invitrogen Life Technologies). Bound Ig-fusion proteins were eluted with 0.1 M citric acid (pH 2.8), and the collected fractions were immediately neutralized with Tris base (pH 10.85). The fractions containing protein were identified by measuring the optical density (A_{280}) and then were pooled, dialyzed against 1×PBS, (Invitrogen Life Technologies) and filtered through a 0.2 μm filter.

[0278] The purified Ig-fusion proteins were analyzed by SDS-PAGE. Aliquots of 2H7 scFv-llama IgG1, 2H7 scFv-llama IgG2, 2H7 scFv-llama IgG3, and Rituxan® (Rituximab, anti-CD20 antibody, Genentech, Inc. and IDEC Pharmaceuticals Corp.) (provided by Dr. Oliver W. Press, Fred Hutchison Cancer Research Center, Seattle, Wash.) (5 μg protein) were combined with 25 μl 2×NuPAGE® SDS Sample Buffer (Invitrogen Life Technologies) (non-reduced samples). Samples of each protein were also prepared in reducing sample buffer containing 5% 2-mercaptoethanol (Sigma-Aldrich, St. Louis, Mo.). Molecular weight markers (Invitrogen Life Technologies) were applied to the gels in non-reducing buffer only. The proteins were fractionated on NuPAGE® 10% Bis-Tris gels (Invitrogen Life Technologies). After electrophoresis (approximately 1 hour), the gels were washed three times, five minutes each, with Distilled Water (Invitrogen Life Technologies) and then stained in 50 ml Bio-Safe Coommassie Stain (BioRad, Hercules, Calif.) overnight at room temperature. After a wash in Distilled Water, the gels were photographed. The migration pattern of each Ig-fusion protein is presented in **FIG. 24**.

[0279] The ability of the 2H7 scFv-llama Ig fusion proteins to bind to cells expressing CD20 was demonstrated by flow cytometry. Serial dilutions starting at 25 μg/ml of purified 2H7 scFv-llama IgG1, 2H7 scFv-llama IgG2, and 2H7 scFv-llama IgG3 were prepared and incubated with CD20-transfected (CD20+) CHO cells (from the laboratory of Dr. S. Skov, Institute of Medical Microbiology and Immunology, Copenhagen Denmark in 1% FBS 1×PBS media (Invitrogen Life Technologies) for one hour on ice. After the incubation, the cells were then centrifuged and washed with 1% FBS in 1×PBS. To detect bound 2H7 scFv-llama Ig, the cells were incubated for one hour on ice with fluorescein-conjugated goat anti-camelid IgG (heavy and light chain) (1:100) (Triple J Farms). The cells were then centrifuged and resuspended in 1% FBS-1×PBS and analyzed using a Coulter Epics XL cell sorter (Beckman Coulter, Miami, Fla.). The data (percent of maximum brightness) are presented in **FIG. 25**.

Example 11

[0280] Effector Function of Anti-CD20 scFv-llama Ig Fusion Proteins

[0281] This Example demonstrates the ability of anti-CD20 llama IgG1, IgG2, and IgG3 fusion proteins to mediate complement dependent cytotoxicity (CDC) and antibody dependent cell-mediated cytotoxicity (ADCC).

[0282] The ability of the 2H7 scFv-llama IgG fusion proteins to kill CD20 positive cells in the presence of complement was tested using the BJAB human B cell line. Rabbit complement was obtained from 3-4 week old rabbits (Pel-Freez, Brown Deer, Wis.). BJAB cells (2×10^6 cells/ml) were combined with rabbit complement (final dilution 1:10) and purified 2H7 Ig fusion proteins. 2H7 scFv-llama IgG1, 2H7 scFv-llama IgG2, 2H7 scFv-llama IgG3, and 2H7 scFv-human IgG1 wild type hinge-CH2-CH3) (Example 1) were added at 1:3 serial dilutions beginning at a concentration of 30 μg/ml. After one hour at 37° C., cell viability was determined by counting live and dead cells by trypan blue exclusion (0.4%) (Invitrogen Life Technologies) using a hemacytometer (Bright-line, Horsham, Pa.). The percent killing was calculated by dividing the number of dead cells by the number of total cells (dead+live cells). The data presented in **FIG. 26** show that all Ig fusion proteins had CDC activity.

[0283] The ADCC activity of the 2H7 scFv-llama IgG fusion proteins was determined using BJAB cells as target cells and human or llama peripheral blood mononuclear cells (PBMC) as effector cells. BJAB cells were pre-incubated for approximately 2 hours with ^{51}Cr (100 μCi) (Amersham Biosciences, Piscataway, N.J.) in fully supplemented IMDM (Invitrogen Life Technologies) containing 15% FBS. The cells were mixed intermittently during the pre-incubation period. Fresh, resting human PBMC were purified from whole blood using Lymphocyte Separation Media (LSM) (ICN Pharmaceuticals, New York, N.Y.). PBMC were combined with labeled BJAB cells (5×10^4 cells per well of 96 well tissue culture plate) at ratios of 25:1, 50:1, and 100:1. To each combination was added 10 μg/ml of purified 2H7 scFv-llama IgG1, 2H7 scFv-llama IgG2, 2H7 scFv-llama IgG3, Rituximab, or no anti-CD20 antibody. The mixtures were incubated for 6 hours at 37° C. Supernatant from each reaction containing ^{51}Cr released from lysed cells was collected onto a LumaPlate-96 filter plate (Packard, Meriden, Conn.), which was dried overnight. The amount of ^{51}Cr was measured by a TopCount NXT plate reader (Packard).

FIG. 27 shows that the 2H7 scFv-llama IgG2 fusion protein was the most effective llama fusion protein in mediating ADCC. Each data point represents the average measurement of triplicate wells.

[0284] ADCC activity was affected by the source of effector cells. Llama PBMC were isolated from llama blood (Triple J Farms) using LSM. Llama effector cells were added at the same ratios to BJAB target cells as described for the ADCC assay using human effector cells. The cells were combined with 10 μg/ml of purified 2H7 scFv-llama IgG1, 2H7 scFv-llama IgG2, 2H7 scFv-llama IgG3, Rituximab, or no anti-CD20 antibody. The results are presented in **FIG. 28**.

Example 12

[0285] Construction and Characterization of scFv Ig Fusion Proteins Expressed on the Cell Surface

[0286] This Example describes a retroviral transfection system for ectopic surface expression of genetically engineered cell surface receptors composed of scFvs that bind costimulatory receptors. The Example also demonstrates the effector function of these various scFv Ig fusion proteins expressed on the surface of target cells.

[0287] The heavy and light chain variable regions were cloned from murine monoclonal antibodies specific for various costimulatory receptors, and single chain Fv constructs were prepared essentially as described in Example 1. Antibodies included 2H7, anti-human CD20; 40.2.220, anti-human CD40; 2E12, anti-human CD28; 10A8, anti-human CD152 (anti-CTLA-4); and 500A2, anti-murine CD3. The heavy chain and light chain variable regions of each antibody were cloned according to standard methods for cloning immunoglobulin genes and as described in Example 1. Single chain Fv constructs were prepared as described in Example 1 by inserting a nucleotide sequence encoding a $(gly_4ser)_3$ peptide linker between the VL region nucleotide sequence of 40.2.220, 2E12, 10A8, and 500A2, respectively (SEQ ID NOs:______, respectively) and the VH region nucleotide sequence of 40.2.220, 2E12, 10A8, and 500A2, respectively (SEQ ID NOs:______, respectively). The polypeptide sequence for VL of 40.2.220, 2E12, 10A8, and 500A2 are set forth in SEQ ID NOs:______, respectively, and the polypeptide sequence for VH of 40.2.220, 2E12, 10A8, and 500A2 are set forth in SEQ ID NOs:______, respectively. Each scFv polynucleotide (SEQ ID NOs:______ for 40.2.220, 2E12, 10A8, and 500A2, respectively) was then fused to human IgG1 mutant hinge (CCC→SSS) and mutant CH2 (proline to serine mutation at residue 238 (238 numbering according to EU nomenclature, Ward et al., 1995 *Therap. Immunol.* 2:77-94; residue 251 according to Kabat et al.) and wild type CH3 domains according to the methods described in Example 5 and 11. Each scFv mutant IgG1 fusion polynucleotide sequence was then fused in frame to sequences encoding the transmembrane domain and cytoplasmic tail of human CD80 (SEQ ID NO:______), such that when the fusion protein was expressed in the transfected cell, CD80 provided an anchor for surface expression of the scFv Ig fusion protein. cDNAs encoding the scFv-IgG-CD80 fusion proteins (SEQ ID NOs:______ for 40.2.220-, 2E12-, 10A8-, and 500A2-scFv-IgG-CD80, respectively) were inserted into the retroviral vector pLNCX (BD Biosciences Clontech, Palo Alto, Calif.) according to standard molecular biology procedures and vendor instructions. The scFv-Ig-CD80 cDNA was inserted between the 5'LTR-neomycin resistance gene-CMV promoter sequences and the 3'LTR sequence. The retroviral constructs were transfected into Reh, an acute lymphocytic leukemia cell line (ATCC CRL-8286). Transfected cells were screened to select clones that were expressing scFv-Ig fusion proteins on the cell surface.

[0288] CDC and ADCC assays were performed with the transfected Reh cells to determine if expression of the scFv-Ig polypeptides on the cell surface augmented effector cell function. Reh cells expressing anti-human CD152 scFv-mutant IgG-CD80 (SEQ ID NO:______); Reh anti-human CD28 scFv- mutant IgG-CD80 (SEQ ID NO:______); Reh anti-human CD28 scFv-mutant IgG-CD80 (SEQ ID NO:______); Reh anti-human CD40 scFv-mutant IgG-CD80 (SEQ ID NO:______); Reh anti-human CD20 scFv-mutant IgG-CD80 (SEQ ID NO:______) were combined with human PBMC (see Example 11) and rabbit complement (10 μg/ml) for one hour at 37° C. Untransfected Reh cells were included as a control. Viability of the cells was determined by trypan blue exclusion, and the percent of killed cells was calculated (see Example 11). **FIG. 29** shows the effectiveness of the scFv-IgG-CD80 fusion proteins when expressed on the cell surface of tumor cells to mediate complement dependent cytotoxicity.

[0289] The same transfected Reh cells tested in the CDC assay plus Reh cells transfected with the polynucleotide construct that encodes anti-murine CD3-scFv-Ig-CD80 (SEQ ID NO:______) were analyzed for ADCC activity (see Example 11). Untransfected and transfected Reh cells were pre-labeled with ^{51}Cr (100 μCi) (Amersham) for two hours at 37° C. Human PBMC served as effector cells and were added to the Reh target cells (5×10^4 cells per well of 96 well plate) at ratios of 5:1, 2.5:1, and 1.25:1. After five hours at 37° C., culture supernatants were harvested and analyzed as described in Example 11. Percent specific killing was calculated according to the following equation: ((experiment release minus spontaneous release)/(maximum release minus spontaneous release))×100. The data are presented in **FIG. 30**. Each data point represents the average of quadruplicate samples.

[0290] Using the same procedures described above, the same results with other binding domains were obtained using the following monoclonal antibodies mAbs as sources of sFv: for CD20, 1F5 (Genbank AY 058907 and AY058906); for CD40, 2.36 and G28.5; for CD28, 9.3.

[0291] Cell surface expression of antibody binding domains is accomplished by fusing antibody scFvs to IgA hinge and constant regions and IgE hinge and constant regions. Polynucleotides encoding an anti-4-1BB scFv, 5B9 (anti-human 4-1BB) scFv, and 2e12 (anti-human CD40) fused to IgAH IgA T4 (four terminal CH3 residues deleted) fused to the CD80 transmembrane and cytoplasmic domains and IgE Fc regions are shown in SEQ ID NOs:______. The encoded polypeptides are shown in SEQ ID NOs:______.

Example 13

[0292] Construction and Sequence of Human Ig Hinge-CH2-CH3 Mutants and 2H7 Variable Region Mutants

[0293] This Example describes construction of scFv fusion proteins containing mutant human IgG1 and IgA

constant regions. This Example also describes construction of a 2H7 scFv mutant with a single point mutation in the variable heavy chain region. Mutations were introduced into variable and constant region domains according to methods described herein and known in the molecular biology arts. **FIG. 31** presents nomenclature for the Ig constant region constructs.

[0294] The human IgG1 hinge region of the 2H7 scFv human IgG1 hinge-CH2-CH3 fusion proteins was mutated to substitute cysteine residues that in a whole immunoglobulin are involved in forming disulfide bonds between two heavy chain molecules. One mutant, 2H7 scFv fused to a human IgG1 hinge region in which all three cysteine residues were mutated to serine residues (MTH (SSS)), was prepared as described in Example 5 (designated in Example 5 as CytoxB-MHWTG1C (includes wild type IgG1 CH2 and CH3 domains)) (now referred to as 2H7 scFv MTH (SSS) WTCH2CH3) and comprises the polynucleotide sequence SEQ ID NO:______ encoding the polypeptide as set forth in SEQ ID NO______. The polynucleotide sequence encoding this mutant (SEQ ID NO:______) was used as a template to create mutant hinge regions in which the first two cysteine residues were substituted with serine residues (IgG MTH (SSC)). An oligonucleotide was designed to substitute the third serine residue with a cysteine and had the following sequence: 5'-gtt gtt gat cag gag ccc aaa tct tct gac aaa act cac aca tct cca ccg tgc cca gca cct g-3' (HuIgGMHncs3, SEQ ID NO:______). A second mutant was prepared in which the mutant hinge had serine residues substituting the first and third cysteine residues (IgG MTH (SCS)). The sequence of the oligonucleotide to create this mutant was as follows: 5'-gtt gtt gat cag gag ccc aaa tct tct gac aaa act cac aca tgc cca ccg-3' (HuIgGMHncs2, SEQ ID NO:______). A third mutant was prepared with cysteine residues substituted at the second and third positions (IgG MTH (CSS)), also using the IgG MTH (SSS) mutant as template, and an oligonucleotide having the sequence, 5'-gtt gtt gat cag gag ccc aaa tct tgt gac aaa act cac-3' (HuIgGMHncs1, SEQ ID NO:______).

[0295] The oligonucleotides introducing the mutations into the hinge region were combined with template and a 3' oligonucleotide containing an XbaI site (underlined and italicized) (5'-gtt gtt tct aga tca ttt acc cgg aga cag gga gag gct ctt ctg cgt gta g-3' (SEQ ID NO:______)) to amplify the mutant hinge-wild type (WT)-CH2-CH3 sequences by PCR. The IgG MTH CSS and IgG MTH SCS mutant sequences were amplified for 25 cycles with a denaturation profile of 94° C., annealing at 52° C. for 30 seconds, and extension at 72° C. for 30 seconds. The IgG MTH SSC mutant sequence was amplified under slightly different conditions: denaturation profile of 94° C., annealing at 45° C. for 30 seconds, and extension at 72° C. for 45 seconds. The amplified polynucleotides were inserted into the TOPO® cloning vector (Invitrogen Life Technologies) and then were sequenced as described in Example 1 to confirm the presence of the mutation. pD18 vector containing 2H7 scFv was digested to remove the constant region sequences essentially as described in Example 10. The mutant hinge-wild type CH2-CH3 regions were inserted in frame into the digested vector DNA to obtain vectors comprising 2H7 scFv MTH (CSS) WTCH2CH3 encoding DNA (SEQ ID NO:______); 2H7 scFv MTH (SCS) WTCH2CH3 encoding DNA (SEQ ID NO:______); and 2H7 scFv MTH (SSC) WTCH2CH3 encoding DNA (SEQ ID NO:______).

[0296] A mutation of leucine to serine at position 11 in the first framework region of the heavy chain variable region (numbering according to Kabat et al., *Sequences of Proteins of Immunological Interest*, 5th ed. Bethesda, Md.: Public Health Service, National Institutes of Health (1991)) was introduced into the 2H7 scFv MTH (SSS) WTCH2CH3 fusion protein (SEQ ID NO:______). The wild type leucine residue was substituted with serine by site-directed mutagenesis using the oligonucleotide Vhser11: 5'-gga ggt ggg agc tct cag gct tat cta cag cag tct ggg gct gag tcg gtg agg cc-3' (SEQ ID NO:______). The 3'-primer for PCR was huIgG1-3' having the sequence 5'-gtc tct aga cta tca ttt acc cgg aga cag-3' (SEQ ID NO:______) (XbaI site underlined and italicized). After PCR amplification, the fragments were inserted into the TOPO® cloning vector and sequenced to confirm the presence of the VH11 leucine to serine mutation. The 2H7 scFv-IgG MTH (SSS) WTCH2CH3 encoding DNA was shuttled into the PSL1180 cloning vector (Pharmacia Biotech, Inc., Piscataway, N.J.). The construct PSLT1180-2H7 scFv-IgG MTH (SSS) WTCH2CH3 was digested with Sac and XbaI to remove the wild type VH domain and the hinge and CH2 and CH3 domains. The PCR product comprising the VH11 mutant was digested with Sac and XbaI and then inserted into the digested PSL1180 construct according to standard molecular biology procedures. The construct was then digested with Hind III and XbaI, and inserted into the mammalian expression vector pD18 (see methods described in Example 1 and Example 10). The mutant is designated 2H7 scFv VH11SER IgG MTH (SSS) WTCH2CH3 (**FIG. 31**). The polynucleotide sequence is provided in SEQ ID NO:______, and the encoded polypeptide sequence is provided in SEQ ID NO:______.

[0297] Four constructs containing IgA constant region domains were prepared. One construct contained wild type IgA hinge fused to human IgG1 CH2 and CH3 (IgAH IgG WTCH2CH3) (**FIG. 31**). Sequential PCR amplifications were performed to substitute the human IgG1 hinge of the 2H7 scFv construct with nucleotide sequences encoding the IgA hinge. The 5' oligonucleotide primer (huIgA/Gchim5) for the first PCR reaction had the sequence, 5'-cca tct ccc tca act cca cct acc cca tct ccc tca tgc gca cct gaa ctc ctg-3' (SEQ ID NO:______). The primer (huIgAhg-5') for the second PCR reaction to add more IgA specific hinge sequence and add a BclI restriction enzyme site (italicized and underlined) had the sequence, 5'-gtt gtt gat cag cca gtt ccc tca act cca cct acc cca tct ccc caa ct-3' (SEQ ID NO:______). The 3' primer for both amplification steps was huIgG1-3' having the sequence, 5'-gtc tct aga cta tca ttt acc cgg aga cag-3' (SEQ ID NO:______). The sequence of the PCR product was confirmed by TOPO® cloning as described above. The gel-purified fragment was digested with BclI and XbaI and then inserted into the 2H7 scFv-pD18 vector that had been digested BclI and XbaI to remove all the IgG1 constant region domains. Ligation was performed as described in Example 10 to provide a mammalian expression vector comprising the nucleotide sequence (SEQ ID NO:______) encoding a 2H7 scFv IgA hinge-IgG1 CH2-CH3 polypeptide (SEQ ID NO:______).

[0298] A second pD18 mammalian expression vector was constructed that had a polynucleotide sequence (SEQ ID NO:______) that encoded a 2H7 scFv fused to wild type IgA hinge, CH2, and CH3 domains (SEQ ID NO:______). Human IgA constant regions sequences were obtained by

using random primers to reverse transcribe total RNA isolated from human tonsil followed by PCR amplification of the cDNA using sequence specific primers, essentially as described in Example 10. Human IgA hinge-CH2-CH3 nucleotide sequence (SEQ ID NO:______) encoding the IgA-CH2-CH3 polypeptide (IgAH IgACH2CH3, **FIG. 31**) (SEQ ID NO:______) was amplified using the 5' oligonucleotide hulgAhg-5' (SEQ ID NO:(same as above ______) and a 3' oligonucleotide hulgA3' having the sequence, 5'-gtt gtt tct aga tta tca gta gca ggt gcc gtc cac ctc cgc cat gac aac-3' (SEQ ID NO:______). Secretion of a 2H7-IgA hinge-IgA CH2-CH3 polypeptide from transfected mammalian cells required co-expression of human J chain that covalently binds to two IgA CH3 domains via disulfide bonds. Total RNA was isolated from tonsil B cells and was reversed transcribed to generate cDNA as described above. PCR amplification of the nucleotide sequence encoding the J chain was performed with J chain specific primers. The 5' PCR primer, HUJCH5nl, had the sequence, 5'-gtt gtt aga tct caa gaa gat gaa agg att gtt ctt-3' (SEQ ID NO:______), and sequence of the 3' primer, HUJCH3, was 5'-gtt gtt tct aga tta gtc agg ata gca ggc atc tgg-3' (SEQ ID NO:______). The cDNA was cloned into TOPO® for sequencing as described in Example 10. J chain encoding cDNA (SEQ ID NO:______) was then inserted into pD18 and pcDNA3-Hygro (+) (Invitrogen Life Technology) vectors for co-transfection with 2H7 scFv IgA hinge-CH2-CH3 constructs. The J chain has the predicted amino acid sequence set forth in SEQ ID NO:______.

[0299] Secretion of an scFv IgA constant region construct in the absence of J chain was accomplished by engineering a truncated CH3 domain with a deletion of the four carboxy terminal amino acids (GTCY, SEQ ID NO:______) (IgAH IgA-T4, **FIG. 31**), which include a cysteine residue that forms a disulfide bond with the J chain. The IgA hinge-CH2-CH3 nucleotide sequence containing the deletion in CH3 (SEQ ID NO:______) was prepared using a 5' primer (hulgAhg-5') having the sequence 5'-gtt gtt *gat cag* cca gtt ccc tca act cca cct acc cca tct ccc tca act-3' (SEQ ID NO:______) (BclI site is underlined and italicized), and a 3' PCR primer (HUIGA3T1) having the sequence 5'-gtt gtt tct aga tta tca gtc cac ctc cgc cat gac aac aga cac-3' (SEQ ID NO:______). This mutated IgA constant region nucleotide sequence was inserted into a 2H7 scFv pD18 vector as described for the generation of the previous 2H7 scFv-Ig constructs (see Example 1 and this example) that comprises the polynucleotide sequence (SEQ ID NO:______) encoding a 2H7 IgAH IgA-T4 polynucleotide (SEQ ID NO:______).

[0300] A fourth construct was prepared that encoded a 2H7 scFv-IgA constant region fusion protein with a deletion of 14 additional amino acids, most of which are hydrophobic residues, from the carboxy terminus of IgA CH3. The 2H7 scFv-IgAH IgA-T4 encoding polynucleotide was used as template to engineer a deletion of the nucleotide sequence encoding PTHVNVSVVMAEVD (SEQ ID NO:______). The 5' oligonucleotide primer had the sequence 5'-gtt gtt gat cag cca gtt ccc tca act cca cct acc cca tct ccc tca act-3' (SEQ ID NO:______) (BclI site shown as underlined and italicized). The 3' oligonucleotide sequence was 5'-gtt gtt tct aga tta tca ttt acc cgc caa gcg gtc gat ggt ctt-3' (SEQ ID NO:______). The deleted IgA CH3 region was amplified by using the above oligonucleotides to amplify the IgA constant region from RNA isolated from human tonsil such that the cDNA contained the deleted carboxyl terminal encoding region for the 18 amino acids. The IgAH IgA-T18 constant region was inserted into a 2H7 scFv pD18 vector that comprises the polynucleotide sequence (SEQ ID NO:______) encoding a 2H7 IgAH IgA-T18 polynucleotide (SEQ ID NO:______) as described above.

Example 14

[0301] Effector Function of CTLA-4 IgG Fusion Proteins

[0302] The Example compares the effector functions of CTLA-4 Ig fusion proteins in CDC and ADCC assays.

[0303] Two CTLA-4 IgG fusion proteins were constructed. One fusion protein comprises the extracellular domain of CTLA-4 fused to human IgG1 wild type hinge, CH2, and CH3 domains and is designated CTLA-4 IgG WTH (CCC) WTCH2CH3 (SEQ ID NO:______). A pD18 mammalian expression vector comprising a polynucleotide sequence encoding CTLA-4 IgG WTH (CCC) WTCH2CH3 (SEQ ID NO:______) was prepared by fusing in frame the nucleotide sequence encoding the extracellular domain of CTLA-4 (SEQ ID NO:______) (see U.S. Pat. No. 5,844,095) to the nucleotide sequence encoding IgG WTH (CCC) WTCH2CH3 (SEQ ID NO:______) according to the methods described in Examples 1 and 10. The extracellular domain nucleotide sequence also comprises a BclI restriction enzyme site at the 3' end, and a leader peptide nucleotide sequence (SEQ ID NO:______) that encodes an oncoM leader peptide (SEQ ID NO:______). A second CTLA-4 IgG fusion protein, designated CTLA-4 IgG MTH (SSS) MTCH2WTCH3, contained the extracellular domain of CTLA-4 (plus the oncoM leader peptide sequence) fused to a mutant IgG hinge in which all three cysteine residues were replaced with serine residues. The hinge region was fused to a mutant IgG1 CH2 domain that had a mutation at isotype position 238 (EU numbering, Ward et al., supra, (position 251 using numbering according to Kabat et al., supra; position 209 where numbering commences with first residue of IgG1 CH1; i.e., PAPELLDGPS (SEQ ID NO:______) of wild type IgG1 CH2 is modified to PAPELLDGSS (SEQ ID NO:______)), which was fused to IgG1 wild type CH3 (U.S. Pat. No. 5,844,095). The CTLA-4 IgG MTH (SSS) MTCH2WTCH3 polynucleotide comprises the nucleotide sequence in SEQ ID NO:______ and the deduced amino acid sequence comprises the sequence provided in SEQ ID NO:______. CTLA-4 fusion proteins were also prepared using CTLA-4 extracellular membrane encoding sequences without the leader peptide (SEQ ID NO:______).

[0304] To measure CDC activity, purified CTLA-4 IgG WTH (CCC) WTCH2CH3 (2 μg/ml) or CTLA-4 IgG MTH (SSS) MTCH2WTCH3 (2 μg/ml) was added to Reh cells (see Example 12) and to Reh cells transfected with the costimulatory molecule CD80 such that CD80 was expressed on the cell surface (Reh CD80.10, obtained from Dr. E. Clark, University of Washington, Seattle, Wash.; see Doty et al., 1998 *J. Immunol.* 161:2700; Doty et al., 1996 *J. Immunol.* 157:3270), in the presence or absence of rabbit complement (10 μg/ml). Purified CTLA Ig fusion proteins were prepared from culture supernatants of transiently transfected COS cells according to methods described in Example 10. The assays were performed essentially as described in Example 11 and 12. The data presented in **FIG.**

32 show that only CD80-transfected Reh cells were killed in the presence of complement and CTLA-4 IgG WTH (CCC) WTCH2CH3 fusion protein.

[0305] The purified CTLA-4 Ig fusion proteins were also tested in ADCC assays. Human PBMC, serving as effector cells, were added to Reh or Reh CD80.1 target cells at a ratio of 1.25:1, 2.5:1, 5.0:1, and 10:1. Cells were labeled and the assays performed essentially as described in Examples 11 and 12. The results are presented in **FIG. 33**. Each data point represents the average of four independent culture wells at each effector:target cell ratio. The data show that only CTLA-4 IgG WTH (CCC) WTCH2CH3 mediated significant ADCC of Reh CD80.10 cells.

Example 15

[0306] Effector Function of CTLA-4 IgA Fusion Proteins

[0307] CTLA-4 IgA fusion proteins are prepared as described for the IgG fusion proteins (see Examples 1, 13, and 14). CTLA-4 extracellular domain nucleotide sequence (SEQ ID NO:______) is fused in open reading frame to nucleotides encoding IgAH IgACH2CH3 (SEQ ID NO:______) to provide the nucleotide sequence (SEQ ID NO:______) encoding a CTLA-4 IGAH IgACH2CH3 fusion protein (SEQ ID NO:______). The fusion protein is transiently expressed in COS cells (see Example 10) or stably expressed in CHO cells (see Example 1). Secretion of the CTLA-4 IgAH IgACH2CH3 fusion protein requires co-transfection with a construct containing a polynucleotide sequence (SEQ ID NO:______) that encodes human J chain (SEQ ID NO:______). The CTLA-4 IgAH IgACH2CH3 fusion protein is isolated as described in Examples 10 and 14. To express a CTLA-4 IgA construct without the presence of J chain, a CTLA-4 IgAH IgA-T4 construct is prepared and transfected into mammalian cells. In a similar manner as described for the CTLA-4 extracellular fragment fused to wild type IgA hinge-CH2CH3, the CTLA-4 extracellular domain nucleotide sequence (SEQ ID NO:______) is fused in open reading frame to a nucleotide sequence (SEQ ID NO:______) encoding a IgAH IgA-T4 polypeptide (SEQ ID NO:______) to provide a nucleotide sequence comprising SEQ ID NO:______ encoding a CTLA-4 IgAH IgA-T4 polypeptide (SEQ ID NO:______). Effector function of each construct is evaluated by CDC and ADCC as described in Example 14.

Example 16

[0308] Binding of Anti-CD20 scFv Human Ig Fusion Proteins to CHO Cells Expressing CD20

[0309] This Example describes binding of 2H7 scFv Ig fusion proteins to CHO cells that express CD20. The analysis was performed by flow cytometry. Culture supernatants were collected from transiently transfected COS cells expressing 2H7 scFv IgG WTH (CCC) WTCH2CH3 (SEQ ID NO:______); 2H7 scFv IgG MTH (CSS) WTCH2CH3 (SEQ ID NO:______); 2H7 scFv IgG MTH (SCS) WTCH2CH3 (SEQ ID NO:______); and 2H7 scFv VHSER11 WTH WTCH2CH3 (SEQ ID NO:______), and two-fold serial dilutions were prepared. Serial two-fold dilutions of purified 2H7 scFv IgG MTH (SSC) WTCH2CH3 (SEQ ID NO:______) were prepared starting at a concentration of 5 μg/ml. The culture supernatants and purified fusion protein samples were incubated with (CD20+) CHO cells for one hour on ice. The cells were washed twice and then incubated with 1:100 FITC-conjugated goat anti-human IgG (CalTag) for 40 minutes. The unbound conjugate was then removed by washing the cells and flow cytometry analysis was performed using a Coulter Epics XL cell sorter. Results are presented in **FIG. 34**.

Example 17

[0310] Immunoblot Analysis of Anti-CD20 scFv Human IgG and IgA Fusion Proteins

[0311] This Example describes immunoblot analysis of 2H7 scFv IgG and 2H7 scFv IgA fusion proteins that were immunoprecipitated from transfected cell culture supernants.

[0312] COS cells were transiently transfected with plasmids comprising nucleotide sequences for 2H7 scFv IgG WTH (CCC) WTCH2CH3 (SEQ ID NO:); 2H7 scFv IgG MTH (CSS) WTCH2CH3 (SEQ ID NO:); 2H7 scFv IgG MTH (SCS) WTCH2CH3 (SEQ ID NO:); 2H7 scFv IgA H IgG WTCH2CH3 (SEQ ID NO:); and scFv IgG MTH (SSS) WTCH2CH3 (SEQ ID NO:) essentially according to the method described in Example 10. Cells were also transfected with vector only. After 48-72 hours at 37° C., cell culture supernatants were harvested and combined with protein A-agarose beads (Repligen) for one hour at 4° C. The beads were centrifuged and washed several times in TNEN [20 mM Tris base, 100 mM NaCl, 1 mM EDTA, and 0.05% NP-40, pH 8.0). The immunoprecipitates were combined with 25 μl 2xNuPAGE® SDS Sample Buffer (Invitrogen Life Technologies) (non-reduced samples). The proteins were fractionated on NuPAGE® 10% Bis-Tris gels (Invitrogen Life Technologies). After electrophoresis (approximately 1 hour), the proteins were transferred from the gel onto a Immobilon P polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, Mass.) using a semi-dry blotter (Ellard Instrumentation, Monroe, Wash.). The PVDF membrane was blocked in PBS containing 5% nonfat milk and then probed with HRP-conjugated goat anti-human IgG (Fc specific) (CalTag). After washing the immunoblot several times in PBS, the blot was developed using ECL (Amersham Biosciences). The results are shown in **FIG. 35**.

Example 18

[0313] Binding of Anti-CD20 scFv Human IgA Fusion Proteins to CD20+ CHO Cells

[0314] This Example describes flow immunocytofluorimetry analysis of binding of 2H7 scFv IgAH IgACH2CH3 (SEQ ID NO:______) and 2H7 scFv IgAH IgAT4 (SEQ ID NO:______) fusion proteins to (CD20+) CHO cells.

[0315] COS cells were transiently co-transfected as described in Example 10 with plasmid DNA comprising a polynucleotide sequence (SEQ ID NO:______) encoding 2H7 scFv IgAH IgACH2CH3 polypeptide (SEQ ID NO:______) and with a separate plasmid comprising a polynucleotide sequence (SEQ ID NO:______) encoding a human J chain polypeptide (SEQ ID NO:______). COS cells were also transfected with a polynucleotide sequence (SEQ ID NO:______) encoding a 2H7 scFv IgA fusion protein that had a deletion of four amino acids at the carboxy terminus of CH3 (2H7 scFv IgAH IgA-T4, SEQ ID NO:______). The transfections were performed as described in Example 10.

Culture supernatants from transfected COS cells were combined with (CD20+) CHO cells (see Example 1) and incubated for one hour on ice. The cells were washed twice with PBS-2%FBS and then combined with FITC-conjugated goat anti-human IgA chain (CalTag) (1:100) for 40 minutes. The cells were again washed and then analyzed by flow cytometry using a Coulter Epics XL cell sorter. **FIG. 36** shows that co-transfection with J chain was not required for secretion of 2H7 scFv IgAH IgAT4, the 2H7 IgA fusion protein with the truncated CH3 carboxy end (SEQ ID NO:______).

Example 19

[0316] Effector Function of Anti-CD20 scFv Human IgA Fusion Proteins

[0317] This Example illustrates ADCC activity of 2H7 IgG and IgA fusion proteins against cells that express CD20. BJAB cells were prelabeled with ^{51}Cr (100 μCi) (Amersham) for two hours at 37° C. Effector cells were obtained from fresh, resting human whole blood, which was diluted in an equal volume of Alsever's solution to prevent coagulation. 2H7 scFv IgG MTH (SSS) WTCH2CH3 (SEQ ID NO:______); 2H7 scFv IgG MTH (SCS) WTCH2CH3 (SEQ ID NO:______); 2H7 scFv IgG WTH (CCC) WTCH2CH3 (SEQ ID NO:______); and 2H7 scFv IgAH IgACH2CH3 (SEQ ID NO:______) fusion proteins were purified from transiently transfected COS cell supernatants (100-200 ml) by protein A chromatography as described in Example 10. COS cells transfected with the plasmid encoding 2H7 scFv IgAH IgACH2CH3 were co-transfected with a plasmid encoding human J chain as described in Example 18. Two-fold serial dilutions of the purified 2H7 Ig fusion proteins starting at 5 μg/ml were added to the labeled BJAB cells (5×10^4 cells per well of 96 well tissue culture plate) in the presence of whole blood (100 μl of whole blood diluted 1:1 in Alsever's solution, final dilution 1:4) and incubated for five hours at 37° C. Culture supernatants were harvested and analyzed as described in Example 11. Percent specific killing was calculated according to the following equation: ((experiment release minus spontaneous release)/(maximum release minus spontaneous release))×100. The data are presented in **FIG. 37**. Each data point represents the average of quadruplicate samples.

[0318] In a second ADCC assay, the number of labeled BJAB target cells was held constant in each sample, and whole blood was added at dilutions of 0.25, 0.125, and 0.0625. Purified 2H7 IgG and IgA fusion proteins were added at a concentration of 5 μg/ml. The BJAB cells, whole blood, and fusion proteins were incubated, the supernatants harvested, and the percent specific killing was calculated as described above. Percent specific killing for each of the 2H7 fusion proteins is presented in **FIG. 38**.

[0319] The ADCC activity of purified 2H7 scFv IgG MTH (SSS) WTCH2CH3 (5 μg/ml) and of purified 2H7 scFv IgAH IgACH2CH3 (5 μg/ml) was compared in the presence of different effector cell populations. PBMC were isolated from whole blood as described in Examples 11 and 12. PBMC were combined with labeled BJAB target cells (5×10^4 per well of 96 well tissue culture plate) at ratios of 50:1, 25:1, and 12.5:1. The assay was performed and the data analyzed as described above. **FIG. 39A** shows that only the 2H7 scFv IgG MTH (SSS) WTCH2CH3 fusion protein had ADCC activity when PBMC served as the effector cells. **FIG. 39B** shows that both 2H7 scFv IgG MTH (SSS) WTCH2CH3 and 2H7 scFv IgAH IgACH2CH3 exhibit ADCC activity when whole blood was the source of effector cells (as illustrated in **FIG. 38**).

Example 20

[0320] Expression Level of 2H7 scFv VH11Ser IgG MTH (SSS) WTCH2CH3 Fusion Protein

[0321] This Example compares the expression level of 2H7 scFv VH11Ser IgG MTH (SSS) WTCH2CH3 fusion protein (SEQ ID NO:______) with other 2H7 scFv IgG constructs that do not contain the mutation in the variable heavy chain domain. The mammalian expression vector pD18 comprising nucleotide sequences 2H7 scFv IgG MTH (SSS) WTCH2CH3 (SEQ ID NO:______); 2H7 scFv IgG MTH (CSS) WTCH2CH3 (SEQ ID NO:______); 2H7 scFv IgG MTH (SCS) WTCH2CH3 (SEQ ID NO:______); 2H7 scFv IgG WTH (CCC) WTCH2CH3 (SEQ ID NO:______); and 2H7 scFv VHSER11 IgG MTH (SSS) WTCH2CH3 (see Examples 1 and 13) were transiently transfected into COS cells as described in Example 10. After 72 hours at 37° C., culture supernatants were harvested, and 1 μl of each supernatant was combined with non-reducing sample buffer (see method described in Example 10). The culture supernatant samples and aliquots of purified 2H7 scFv IgG MTH (SSS) WTCH2CH3 (40 ng, 20 ng, 10 ng/5 ng, and 2.5 ng) were fractionated on 10% Bis-Tris (MOPS) NuPAGE® gels (Invitrogen Life Technologies). Multimark® protein standards (Invitrogen Life Technologies) were also separated on the gel. The proteins were transferred to a PDVF membrane and immunoblotted as described in Example 17. The immunoblot is presented in **FIG. 40**. The amounts of the fusion proteins were quantified by densitometry analysis of the blots using the ScionImage for Windows software and comparison with the standard curve. The 2H7 scFv IgG WTH (CCC) WTCH2CH3 construct produced approximately 12 ng/ul or 12 micrograms/ml, the 2H7 scFv IgG MTH (CSS) WTCH2CH3 produced approximately 10 ng/ul or 10 micrograms/ml, the 2H7 scFv IgG MTH (SCS) WTCH2CH3 construct produced approximately 1 ng/ul or 1 microgram/ml, and the 2H7 scFv VHSER11 IgG MTH (SSS) WTCH2CH3 construct produced approximately 30 ng/ml or 30 micrograms/ml.

Example 21

[0322] Construction of a 2H7 scFv IgG Fusion Protein with a Mutant CH3 Domain

[0323] Amino acid mutations were introduced into the CH3 domain of a 2H7 IgG fusion protein. The pD18 vector comprising 2H7 scFv IgG MTH (SSS) WTCH2CH3 (SEQ ID NO:______) was digested with BclI and XbaI to remove the MTH WTCH2CH3 (SEQ ID NO:______) fragment, which was then subcloned into pShuttle vector (BD Biosciences Clontech, Palo Alto, Calif.) that was double-digested with BclI and XbaI. Subcloning was performed in a kanamycin resistant vector because the ampicillin resistance gene has an XmnI site, which is required for this cloning procedure. Five constructs were prepared with the following substitutions: (1) a phenylalanine residue at position 405 (numbering according to Kabat et al. supra) was substituted with tyrosine using the oligonucleotide CH3Y405; (2) the phenylalanine position at 405 was substituted with an ala-

nine residue using the oligonucleotide CH3A405; (3) the tyrosine residue at position 407 was substituted with an alanine using the oligonucleotide CH3A407; (4) both wild type amino acids at positions 405 and 407 were substituted with tyrosine and alanine, respectively using the oligonucleotide CH3Y405A407; and (5) both wild type amino acids at positions 405 and 407 were substituted with alanine using the oligonucleotide CH3A405A407. The oligonucleotides were the 3' primers for PCR amplification of a portion of the CH3 domain. The nucleotide sequences for each 3' oligonucleotide were as follows.

```
CH3Y405:     5'-gtt gtt gaa gac gtt ccc ctg ctg cca cct gct ctt gtc cac ggt gag ctt gct   (SEQ ID NO:__)
gta gag gta
gaa gga gcc-
3'

CH3A405:     5'-gtt gtt gaa gac gtt ccc ctg ctg cca cct gct ctt gtc cac ggt gag ctt gct   (SEQ ID NO:__)
gta gag ggc
gaa gga gcc-
3'

CH3A407:     5'-gtt gtt gaa gac gtt ccc ctg ctg cca cct gct ctt gtc cac ggt gag ctt gct   (SEQ ID NO:__)
ggc gag gaa
gaa gga gcc-
3'

CH3Y405A407: 5'-gtt gtt gaa gac gtt ccc ctg ctg cca cct gct ctt gtc cac ggt gag           (SEQ ID NO:__)
ctt gct ggc
gag gta gaa
gga gcc-3'

CH3A405A407: 5'-gtt gtt gaa gac gtt ccc ctg ctg cca cct gct ctt gtc cac ggt gag           (SEQ ID NO:__)
ctt gct ggc
gag ggc gaa
gga gcc-3'
```

[0324] The template was the mutant hinge MHWTCH2CH3 human IgG1. The 5' PCR oligonucleotide primer was huIgGMHWC, [SEQ ID NO:______]. The amplified products were TOPO® cloned and sequenced as described in Examples 1 and 10. DNA from the clones with the correct sequence was digested with BclI and XmnI and transferred to pShuttle containing the MTH WTCH2CH3 sequence, which was also digested with the same restriction enzymes. The mutated IgG sequences were then removed by digestion with BclI and XbaI and inserted into a pD18 vector containing 2H7 scFv that was also digested with BclI and XbaI. The polynucleotide sequences for mutated the CH3 domains, MTCH3 Y405, MTCH3 A405, MTCH3 A407, MTCH3 Y405A407, and MTCH3 A405A407 are shown in SEQ ID NOs:______, respectively, and the polypeptide sequences for each are shown in SEQ ID NOs:______, respectively. The polynucleotide sequences for the 2H7 scFv MTH WTCH2 MTCH3 Y405, 2H7 scFv MTH WTCH2 MTCH3 A405, scFv MTH WTCH2 MTCH3 A407, scFv MTH WTCH2 MTCH3 Y405A407, and scFv MTH WTCH2 MTCH3 A405A407, respectively, and the deduced amino acid sequences are shown in SEQ ID NOs:______, respectively.

Example 22

[0325] Construction of 2H7 scFv IgG Fusion Proteins with Hinge Mutations

[0326] A 2H7 scFv IgG fusion protein was constructed with the third cysteine residue in the IgG1 hinge region substituted with a serine residue. The template for introduction of the mutations was a polynucleotide encoding 2H7 scFv WTH WTCH2CH3 (SEQ ID NO:______). The oligonucleotide introducing the mutations was a 5' PCR primer oligonucleotide HIgGMHcys3 having the sequence 5'-gtt gtt gat cag gag ccc aaa tct tgt gac aaa act cac aca tgt cca ccg tcc cca gca cct-3'. The oligonucleotide introducing the mutation into the hinge region was combined with template and a 3' oligonucleotide containing an XbaI site (underlined and italicized) (5'-gtt gtt <u>tct aga</u> tca ttt acc cgg aga cag gga gag gct ctt ctg cgt gta g-3' (SEQ ID NO:______)) to amplify the mutant hinge-wild type (WT)-CH2-CH3 sequences by PCR. The IgG MTH CCS mutant sequence was amplified for 30 cycles with a denaturation profile of 94° C., annealing at 50° C. for 30 seconds, and extension at 72° C. for 30 seconds. The amplified polynucleotides were inserted into the TOPO® cloning vector (Invitrogen Life Technologies) and then were sequenced as described in Example 1 to confirm the presence of the mutation. pD18 vector containing 2H7 scFv was digested to remove the constant region sequences essentially as described in Example 10. The mutant hinge-wild type CH2-CH3 regions were inserted in frame into the digested vector DNA to obtain vectors comprising 2H7 scFv MTH (CCS) WTCH2CH3 encoding DNA (SEQ ID NO:______). The deduced polypeptide sequence is shown in SEQ ID NO:______.

Example 23

[0327] Construction of Anti-CD20 IgE Fusion Proteins

[0328] A binding domain is fused to IgE constant region sequences such that the expressed polypeptide is capable of inducing an allergic response mechanism. The single chain Fv nucleotide sequence of 40.2.220 (SEQ ID NO:______), an anti-CD40 antibody, is fused to IgE CH2-CH3-CH4 according to methods described for other scFv immunoglobulin constant region constructs (see Examples 1, 5, 10, and 13). To PCR amplify the IgE CH2-CH3-CH4 domains, a 5' oligonucleotide primer, hIgE5Bcl, having the sequence 5'-gtt gtt gat cac gtc tgc tcc agg gac ttc acc cc-3', and a 3' oligonucleotide primer, hIgE3stop, having the sequence 5'-gtt gtt tct aga tta act ttt acc <u>ggg</u> att tac aga cac cgc tcg ctg g-3' are used.

[0329] The retroviral transfection system for ectopic surface expression of genetically engineered cell surface receptors composed of scFvs that bind costimulatory receptors described in Example 12 is used to construct a 40.2.220 scFv IgE-CD80 fusion protein. The 40.2.220 scFv IgE fusion polynucleotide sequence is fused in frame to sequences encoding the transmembrane domain and cytoplasmic tail of human CD80 (SEQ ID NO:______), such that when the fusion protein is expressed in the transfected cell, CD80 provided an anchor for surface expression of the scFv Ig fusion protein. cDNA encoding the anti-CD40 scFv-IgE-CD80 fusion proteins (SEQ ID NO:______) is inserted into the retroviral vector pLNCX (BD Biosciences Clontech) according to standard molecular biology procedures and vendor instructions. The 40.2.220 scFv-Ig-CD80 cDNA is inserted between the 5'LTR-neomycin resistance gene-CMV promoter sequences and the 3'LTR sequence. The retroviral constructs are transfected into a carcinoma cell line, and transfected cells are screened to select clones that are expressing the 40.2.220 scFv-Ig-CD80 fusion protein on the cell surface.

Example 24

[0330] Construction of IgA-T4 Mutants that are Expressed on the Cell Surface

[0331] The retroviral transfection system for ectopic surface expression of genetically engineered cell surface receptors composed of scFvs that bind costimulatory receptors described in Example 12 is used to construct a 2H7 scFv IgA hinge IgA-T4-CD80 fusion protein. The 2H7 scFv IgAH IgA-T4 fusion polynucleotide sequence (SEQ ID NO:______) is fused in frame to sequences encoding the transmembrane domain and cytoplasmic tail of human CD80 (SEQ ID NO:______), such that when the fusion protein is expressed in the transfected cell, CD80 provided an anchor for surface expression of the scFv Ig fusion protein. cDNA encoding the 2H7 scFv IgAH IgA-T4-CD80 fusion protein (SEQ ID NO:______) is inserted into the retroviral vector pLNCX (BD Biosciences Clontech) according to standard molecular biology procedures and vendor instructions. The 2H7 scFv IgAH IgA-T4-CD80 cDNA is inserted between the 5'LTR-neomycin resistance gene-CMV promoter sequences and the 3'LTR sequence. The retroviral construct is transfected into Reh, an acute lymphocytic leukemia cell line (ATCC CRL-8286). Transfected cells are screened to select clones that are expressing 2H7 scFv-Ig fusion proteins on the cell surface.

Example 25

[0332] Characterization of an Anti-4-1BB scFv Ig-CD80 Fusion Protein Expressed on the Cell Surface of Tumor Cells and Growth of the Tumor Cells in Vivo

[0333] This example describes construction of an anti-murine 4-1BB (CD137) scFv fusion protein that has an IgG wild type hinge and CH2 and CH3 domains that is fused to the CD80 transmembrane and cytoplasmic domains. The Example also illustrates the effect of the cell surface expression of the anti-4-1BB scFv IgG CD80 polypeptide when the transfected tumor cells are transplanted into mice.

[0334] The heavy and light chain variable regions of a rat anti-4-1BB (CD137) monoclonal antibody (1D8) were cloned, and a single chain Fv construct was prepared essentially as described in Example 1. The heavy chain and light chain variable regions of each antibody were cloned according to standard methods for cloning immunoglobulin genes and as described in Example 1. Aingle chain Fv construct was prepared as described in Example 1 by inserting a nucleotide sequence encoding a $(gly_4ser)_3$ peptide linker between the VL region nucleotide sequence of 1D8 (SEQ ID NO:______) and the VH region nucleotide sequence of 1D8 (SEQ ID NO:______). The polypeptide sequence for 1D8 VL is shown in SEQ ID NO:______), and the polypeptide sequence for the VH domain is shown in SEQ ID NO:______). The scFv polynucleotide (SEQ ID NO:______) was then fused to human IGG1 wild-type hinge-CH2-CH3 domains according to the methods described in Example 1. The scFv IgG1 fusion polynucleotide sequence was then fused in frame to sequences encoding the transmembrane domain and cytoplasmic tail of human CD80 (SEQ ID NO:______) essentially as described in Example 12, such that when the fusion protein was expressed in the transfected cell, CD80 provided an anchor for surface expression of the scFv Ig fusion protein. cDNA encoding the scFv-IgG-CD80 fusion protein (SEQ ID NO:______) was inserted into the retroviral vector pLNCX (BD Biosciences Clontech) according to standard molecular biology procedures and vendor instructions. The scFv-Ig-CD80 cDNA was inserted between the 5'LTR-neomycin resistance gene-CMV promoter sequences and the 3'LTR sequence.

[0335] The retroviral constructs were transfected into the metastatic M2 clone of K1735, a melanoma cell line, provided by Dr. I. Hellstrom, PNRI, Seattle, Wash. Transfected cells were screened to select clones that were expressing scFv-Ig fusion proteins on the cell surface. To demonstrate that the 1D8 scFv IgG-CD80 construct was expressed on the cell surface of the tumor cells, the transfected cells were analyzed by flow immunocytofluorimetry. Transfected cells (K1735-1D8) were incubated for one hour on ice in phycoerythrin-conjugated $F(ab')_2$ goat anti-human IgG. The unbound conjugate was then removed by washing the cells and flow cytometry analysis was performed using a Coulter Epics XL cell sorter. Results are presented in **FIG. 41A**.

[0336] The growth of K1735-1D8 transfected cells was examined in vivo. K1735-WT cells grew progressively when transplanted subcutaneously (s.c.) in naïve C3H mice. Although the same dose of K1735-1D8 cells initially formed tumors of an approximately 30 mm^2 surface area, the tumors started to regress around day 7 and had disappeared by day 20 as shown in **FIG. 41B**. Tumor cells that were transfected with a similarly constructed vector encoding a non-binding scFv, a human anti-CD28 scFv construct, grew as well as tumor cells that had not been transfected. The presence of a foreign protein, that is, human IgG1 constant domains or rat variable regions, did not make transfected K1735-WT cells immunogenic; the growth of the K1735-1D8 cells in C3H mice was identical to that of K1735-WT cells (untransfected).

[0337] To investigate the roles of $CD4^+$ and $CD8^+$ T lymphocytes and NK cells in the regression of K1735-1D8 tumors, naïve mice were injected intraperitoneally (i.p.) with monoclonal antibodies (mAbs, typically 50 μg in a volume 0.1 ml) to remove $CD8^+$, $CD4^+$ or both $CD4^+$ and $CD8^+$ T cells, or were injected with anti-asialo-GM1 rabbit antibodies to remove NK cells. Twelve days later, when flow

cytometry analysis of spleen cells from identically treated mice showed that the targeted T cell populations were depleted, K1735-1D8 cells were transplanted s.c to each T cell-depleted group. K1735-1D8 had similar growth kinetics in mice that had been injected with the anti-CD8 MAb or control rat IgG while removal of $CD4^+$ T cells resulted in the growth of K1735-1D8 with the same kinetics as K1735-WT. This failure to inhibit tumor growth after CD4+ T cell removal was observed regardless of the presence or absence of CD8+ T cells. K1735-1D8 grew in all NK-depleted mice, although more slowly than in the CD4-depleted group. The results are presented in **FIG. 41C**.

Example 26

[0338] Therapeutic Effect of Tumor Cells Expressing Anti-4-1BB scFv IgG-CD80 Fusion Protein

[0339] This Example examines the ability of K1735-1D8 transfected tumor cells expressing an anti-CD137 scFv on the cell surface to generate a sufficient immune response in mice to mediate rejection of established, untransfected wild type tumors. C3H mice were transplanted with K1735-WT tumors (2×10^6 cells/animal) and grown for approximately six days. Experiments were performed using mice with established K1735-WT tumors of 30 mm^2 surface area. Mice were vaccinated by s.c. injection of K1735-1D8 or irradiated K1735-WT cells on the contralateral side. Identical injections were repeated at the time points indicated in **FIG. 42**. One group of animals was given four weekly injections of K1735-1D8 cells. According to the same schedule, another group was given irradiated (12,000 rads) K1735-WT cells, and a third group was injected with PBS. The data are plotted in **FIG. 42**. The WT tumors grew progressively in all control mice and in all mice that received irradiated K1735-WT cells. In contrast, the tumors regressed in 4 of the 5 mice treated by immunization with K1735-1D8. The animals remained tumor-free and without signs of toxicity when the experiment was terminated 3 months later. In the fifth mouse, the tumor nodule decreased in size as long as the mouse received K1735-1D8 cells, but the tumor grew back after therapy was terminated.

[0340] In another experiment with 5 mice/group, mice were injected intravenously (i.v.) with 3×10^5 K1735-WT cells to initiate lung metastases. Three days later, K1735-1D8 cells were transplanted s.c. This procedure was repeated once weekly for a month; control mice were injected with PBS. The experiment was terminated when one mouse in the control group died, 37 days after receiving the K1735-WT cells. At that time, lungs of the control mice each had >500 metastatic foci. In contrast, less than 10 metastatic foci were present in the lungs of the immunized mice.

[0341] In a third experiment, mixtures of K1735-WT cells and K1735-1D8 cells were injected into immunocompetent syngeneic C3H mice. Mice were injected subcutaneously with K7135-WT cells alone or with a mixture of 2×10^6 K1735-WT cells and 2×10^5 K1735-1D8 cells. Tumor growth was monitored at 5-day intervals.

Example 27

[0342] Expression of Anti-4-1BB scFv IgG-CD80 Fusion Protein on the Cell Surface of Sarcoma Cells

[0343] This Example demonstrates expression of an anti-CD137 scFv on the cell surface of a second type of tumor cell by transfecting a murine sarcoma cell line with an anti-CD137 scFv IgG-CD80 construct.

[0344] The 1D8 scFv IgG WTH WTCH2CH3-CD80 polynucleotide (SEQ ID NO:_____) was transferred from the pLNCX vector into pcDNA3-hygro vector using restriction enzyme digestion and ligation steps according to standard molecular biology methods. The constuct was cut with HindIII+ClaI and thesFv fragment was filled in with Klenow (Roche) and the blunt-ended fragment was ligated into EcoR5 site of pcDNA3. Ag104 murine sarcoma tumor cells were transfected with the pcDNA3-hygro vector containing the 1D8 scFv IgG CD80 fusion protein. Hygromycin-resistant clones were screened by flow cytometry using a FITC anti-human IgG antibody to detect expression of the transgene. Only approximately 15% of the resistant clones had detectable fusion protein initially. Positive cells identified by flow cytometry were repeatedly panned on flasks coated with immobilized anti-human IgG (10 μg/ml) according to standard methods. Panning was performed by incubating cells on the coated plates for 30 min at 37 C; the plates were then washed 2-3x in versene or PBS. After each round, cells were tested for IgG expression by FACS. The histogram in **FIG. 44** shows the staining pattern after four rounds of panning against anti-human IgG (black). Untransfected cells were stained and are indicated in gray. All of the cells in the population were positive.

Example 28

[0345] Construction and Characterization of a Bispecific scFv Ig Fusion Protein and scFv Ig Fusion Proteins with a Mutation in the IgG1 CH2 Domain

[0346] An anti-CD20 (2H7) scFv IgG fusion protein was constructed that had a mutant hinge (MT (SSS)) and a mutant CH2 domain in which the proline at residue (position number 238 according to Ward et al., supra) was substituted with a serine. The 2H7 scFv IgG MTH (SSS) MTCH2WTCH3 encoding polynucleotide (SEQ ID NO:______) was constructed essentially according to methods described in Examples 1, 5, and 13. The IgG mutant hinge-mutant CH2-wild type CH3 domains were also fused to an anti-CD20 (2H7)-anti-CD40 (40.2.220) bispecific scFv. The anti-CD20-anti-CD40 scFv IgG MTH (SSS) MTCH2WTCH3 encoding polynucleotide sequence is shown in SEQ ID NO:_____ and the encoded polypeptide is shown in SEQ ID NO:_____.

[0347] COS cells were transiently transfected with vectors comprising the polynucleotide sequences encoding 2H7 scFv IgG MTH (SSS) MTCH2WTCH3 (SEQ ID NO:______); anti-CD20-anti-CD40 scFv IgG MTH (SSS) MTCH2WTCH3 (SEQ ID NO:______); 2H7 scFv IgG MTH (SSS) WTCH2CH3 (SEQ ID NO:______); and 2H7 scFv IgAH IgG WTCH2CH3 (SEQ ID NO:______) as described in Example 10. Culture supernatants were collected and the fusion proteins were purified by protein A chromatography (see Example 10). The purified polypeptides were fractionated by SDS-PAGE according to the method described in Example 10. Rituximab (anti-CD20 monoclonal antibody), and Bio-Rad prestained molecular weight standards (Bio-Rad, Hercules, Calif.), and Multi-

mark® molecular weight standards (Invitrogen Life Technologies were also applied to the gel. The results are presented in **FIG. 45**.

[0348] The 2H7 scFv Ig fusion protein that contains a mutation in the CH2 domain was compared to fusion proteins that have the wild type CH2 domain in an ADCC assay. The assays were performed essentially as described in Examples 11 and 19. Fresh resting PBMC (effector cells) were added to ^{51}Cr-labeled BJAB cells (target cells) at the ratios indicated in **FIG. 46**. Purified 2H7 scFv IgG MTH (SSS) MTCH2WTCH3, 2H7 scFv IgG MTH (SSS) WTCH2CH3, 2H7 scFv IgAH IgG WTCH2CH3, and Rituximab, each at 10 μg/ml were added to the effector/target cell mixtures and incubated for five hours at 37° C. Supernatants were harvested and the amount of chromium released was determined as described in Examples 11 and 19. Percent specific killing by each fusion protein is presented in **FIG. 46**.

Example 29

[0349] Tumor Cell Surface Expression of an Anti-human CD3 scFv IgG Fusion Protein

[0350] An anti-human CD3 scFv Ig CD80 fusion protein was prepared essentially as described in Examples 1 and 12. The fusion protein comprised an anti-human CD3 scFv fused to wild type IgG1 hinge (SEQ ID NO:______) and wild type CH2 (SEQ ID NO:______) and CH3 (SEQ ID NO:______) domains, fused to CD80 transmembrane and cytoplasmic domains (SEQ ID NO:______) to enable cell surface expression of the anti-CD3 scFv. The anti-human CD3 scFv IgG WTH WTCH2CH3-CD80 polynucleotide (SEQ ID NO:______) encoding the polypeptide (SEQ ID NO:______) was transfected in Reh cells and into T51 cells (lymphoblastoid cell line). Expression of the anti-human CD3 scFv IgG fusion protein was detected by flow cytometry using FITC conjugated goat anti-human IgG (see methods in Examples 4, 10, 16, 18). **FIG. 47A** illustrates expression of the anti-human CD3 fusion protein on the cell surface of Reh cells, and **FIG. 47B** shows expression of the fusion protein on T41 cells.

[0351] ADCC assays were performed with the transfected Reh and T51 cells to determine if expression of the scFv-Ig polypeptides on the cell surface augmented effector cell function. Untransfected and transfected Reh cells and untransfected and transfected T51 cells were pre-labeled with ^{51}Cr (100 μCi) (Amersham) for two hours at 37° C. Human PBMC served as effector cells and were added to the target cells (5x10^4 cells per well of 96 well plate) at ratios of 20:1, 10:1, 5:1, and 2.5:1. After four hours at 37° C., culture supernatants were harvested and analyzed as described in Examples 11 and 12. Percent specific killing was calculated as described in Example 12. The results are presented in **FIG. 48**.

Example 30

[0352] Induction of Cytokine Expression in Tumor Cells Expressing Anti-CD28 scFv on the Cell Surface

[0353] This Example describes the effect of cell surface expressed scFv on cytokine mRNA induction in stimulated lymphocytes co-cultured with tumor cells transfected with an anti-human CD28 scFv IgG-CD80 fusion protein.

[0354] Real time PCR analysis was performed on RNA samples from human PBMC stimulated with Reh, Reh-anti-CD28 (2e12) (see Example 12 for construction of 2e12 scFv IgG WTH WHTCH3CH2-CD80 and transfection of Reh cells), and Reh-CD80 (see Example 14) in order to measure the effects of the surface expressed scFv on cytokine production by the PBMC effector cells. For the real-time PCR assay, SYBR Green (QIAGEN) (Morrison et al., *Biotechniques* 24:954-8, 960, 962 (1998)) was used and measured by an ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif.) that measures the formation of PCR product after each amplification cycle. Cells were harvested from cultures and total RNA prepared using QIAGEN RNA kits, including a QIA shredder column purification system to homogenize cell lysates, and RNeasy® mini-columns for purification of RNA. cDNA was reverse transcribed using equal amounts of RNA from each cell type and Superscript II Reverse Transcriptase (Life Technologies). SYBR Green real-time PCR analysis was then performed using the prepared cDNA as template and primer pairs specific for cytokine gene products. The average length of the PCR products that were amplified ranged from 150-250 base pairs. The cDNA levels for many activation response molecules including IFNγ, TNFα, GM-CSF, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-15, ICOSL, CD80 and CD86 were assayed. Control reference cDNAs for constitutively expressed genes, including GAPDH, β-actin, and CD3ε were measured in each assay. The most significant induction of specific mRNA was observed for IFN-γ, and more modest induction was observed for CTLA-4 and ICOS.

Example 31

[0355] Cloning of an Anti-Human 4-1BB Antibody and Construction of an Anti-Human 4-1BB scFv Ig Fusion Protein

[0356] A hybridoma cell line expressing a mouse anti-human monoclonal antibody (designated 5B9) was obtained from Dr. Robert Mittler, Emory University Vaccine Center, Atlanta, Ga. The variable heavy and light chain regions were cloned according to known methods for cloning of immunoglobulin genes and as described herein. Cells were grown in IMDM/15% FBS (Invitrogen Life Technologies) media for several days. Cells in logarithmic growth were harvested from cultures and total RNA prepared using QIAGEN RNA kits, including a QIA shredder column purification system to homogenize cell lysates, and RNeasy® mini-columns for purification of RNA according to manufacturer's instructions. cDNA was reverse transcribed using random hexamer primers and Superscript II Reverse Transcriptase (Invitrogen Life Technologies).

[0357] cDNA was anchor-tailed using terminal transferase and dGTP. PCR was then performed using an anchor-tail complementary primer and a primer that annealed specifically to the antisense strand of the constant region of either mouse Ck (for amplification of VL) or the appropriate isotype mouse CH1 (for amplification of VH). The amplified variable region fragments were TOPO® cloned (Invitrogen Life Technologies), and clones with inserts of the correct size were then sequenced. Consensus sequence for each variable domain was determined from sequence of at least four independent clones. The 5B9 VL and VH polynucleotide sequences are shown in SEQ ID NOs:______ and ______, respectively, and the deduced amino acid sequences are shown in SEQ ID NOs:______ and ______. The scFv was constructed by a sewing PCR method using overlapping

primers containing a synthetic $(gly_4ser)_3$ linker domain inserted between the light and heavy chain variable regions (see Example 1). The 5B9 scFv polypeptide (SEQ ID NO:______) is encoded by the polynucleotide sequence comprising SEQ ID NO:______.

[0358] 5B9 scFv polynucleotide sequence was fused in frame to the polynucleotide sequence encoding the human IgG1 mutant hinge and wild type CH2 and CH3 (MTH (SSS) WTCH2CH3, SEQ ID NO:______) according to methods described in Examples 5, 10, and 13. COS cells were transiently transfected with a vector comprising the 5B9 scFv IgG MTH (SSS) WTCH2CH3 polynucleotide sequence (SEQ ID NO:______). Supernatant was collected and binding of the 5B9 scFv IgG MTH (SSS) WTCH2CH3 polypeptide (SEQ ID NO:______) was measured by flow immunocytofluorimetry essentially as described in Examples 4, 10, 16, and 18. Culture supernatant from the 5B9 hybridoma cell line was also included in the binding assay. Fresh human PBMC were incubated in the presence of immobilized anti-CD3 for four days prior to the binding experiment to induce expression of CD137 on the surface of activated T cells. Stimulated PBMC were washed and incubated with COS or hybridoma culture supernatant containing the 5B9 scFv IgG fusion protein or 5B9 murine monoclonal antibody, respectively, for 1 hour on ice. Binding of 5B9 scFv IgG fusion protein or 5B9 murine monoclonal antibody was detected with FITC conjugated anti-human IgG or anti-mouse IgG, respectively. The results are presented in **FIG. 49**.

Example 32

[0359] Construction of 2H7 scFv IgG Fusion Proteins with Hinge Mutations

[0360] A 2H7 scFv IgG fusion proteins are constructed with the first cysteine residue and the second cystein in the IgG1 hinge region substituted with a serine residue to provide MTH (SCC) and MTH (CSC). The template for introduction of the mutations is a polynucleotide encoding 2H7 scFv WTH WTCH2CH3 (SEQ ID NO:______). The oligonucleotide introducing the mutations are 5' PCR primer oligonucleotides HIgGMHcys1 (SEQ ID NO:______) and HIgGMHcys2 (SEQ ID NO:______). The constructs are prepared as described in SEQ ID NO:______). The encoding polynucleotides of the mutants are presented in SEQ ID NOs:______) and the polypeptide sequences are provided in SEQ ID NO:______).

[0361] Additional representative sequences of the present invention are as follows:

```
HuIgGi wild type hinge, CH2, CH3
tctgatcaggagcccaaatcttgtgacaaaactcacacatgcccaccgtgcccagcacctgaactcctggggggaccgtcagtctt

cctcttccccccaaaacccaaggacaccctcatgatctcccggacccctgaggtcacatgcgtggtggtggacgtgagccacgaa

gaccctgaggtcaagttcaactggtacgtggacggcgtggaggtgcataatgccaagacaaagccgcgggaggagcagtacaa

cagcacgtaccgtgtggtcagcgtcctcaccgtcctgcaccaggactggctgaatggcaaggagtacaagtgcaaggtctccaac

aaagccctcccagcccccatcgagaaaacaatctccaaagccaaagggcagccccgagaaccacaggtgtaCaCcctgccccc

atcccgggatgagctgaccaagaaccaggtcagcctgacctgcctggtcaaaggcttctatcccagcgacatCgcCgtggagtgg

gagagcaatgggcagccggagaacaactacaagaccacgcctcccgtgctggactccgacggctccttcttcctctacagcaag

ctcaccgtggacaagagcaggtggcagcaggggaacgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacgc

agaagagcctctccctgtctccgggtaaatgatctaga

HuIgGI wild type hinge, CH2, CH3
sdqepkscdkthtcppcpapellggpsvflfppkpkdtlmisrtpevtcvvvdvshedpevkthwyvd

gvevhnaktkpreeqynstyrvvsvltvlhqdwlngkeykckvsnkalpapiektiskakgqprepqvytlppsrdeltknqv

sltclvkgfypsdiavewesngqpennykttppvldsdgsfflyskltvdksrwqqgnvfscsvmhealhnhytqkslslspg

k

Llama IgG1 hinge, CH2, CH3
tgatcaagaaccacatggaggatgcacgtgcccncagtgcccncaatgcccngcnccngaactnccagg

aggcccttctgtctttgtcttcccccgaaacccaaggacgtcctctccattttggaggccgagtcacgtgcgttgtagtggacgtcg

gaaagaaagaccccgaggtcaatttcaactggtatattgatggcgttgaggtgcgaacggccaatacgaagccaaaagaggaac

agttcaacagcacgtaccgcgtggtcagcgtcctgcccatccagcaccaggactggctgacggggaaggaattcaagtgcaagg

tcaacaacaaagctctcccggcccccatcgagaggaccatctccaaggccaaagggcagacccgggagccgcaggtgtacacc

ctggccccacaccgggaagaactggccaaggacaccgtgagcgtaacatgcctggtcaaaggcttctacccagctgacatcaac

gttgagtggcagaggaacggtcagccggagtcagagggcacctacgccaacacgccgccacagctggacaacgacgggacct

acttcctctacagcaagctctcggtgggaaagaacacgtggcagcggggagaaaccttaacctgtgtggtgatgcatgaggccct
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-continued

gcacaaccactacacccagaaatccatcacccagtcttcgggtaaatagtaatctaga

Llama IgG1 hinge, CH2, CH3 (In FIG. 23 as Llama IgG1)
ephggctcpqcpapelpggpsvfvtbpkpkdvlsisgrpevtcvvvdvgkedpevnfhwyidgvevr

tantkpkeeqfnstyrvvsvlpiqhqdwltgketkckvnnkalpapiertiskakggqtrepqvytlaphreelakdtvsvtclvk

gfypadinvewqmgqpesegtyantppqldndgtyflysrlsvgkntwqrgctltgvvmhealhnhytqksitqssgk

Llama IgG2:
tgatcaagaacccaagacaccaaaaccacaaccacaaccacaaccacaacccaatcctacaacagaatccaagtgtcccaaatgt

ccagcccctgagctcctgggagggccctcagtcttcatcttcccccgaaacccaaggacgtcctctccatttctgggaggcccga

ggtcacgtgcgttgtggtagacgtgggccaggaagaccccgaggtcagtttcaactggtacattgatggcgctgaggtgcgaacg

gccaacacgaggccaaaagaggaacagttcaacagcacgtaccgcgtggtcagcgtcctgcccatccagcaccaggactggct

gacggggaaggaattcaagtgcaaggtcaacaacaaagctctcccggcccccatcgagaagaccatctccaaggccaaagggc

agacccgggagccgcaggtgtacaccctggccccacaccgggaagagctggccaaggacaccgtgagcgtaacatgcctggt

caaaggcttctacccacctgatatcaacgttgagtggcagaggaatgggcagccggagtcagagggcacytacgccaccacgcc

accccagctggacaacgacgggacctacttcctctacagcaagctctcggtgggaaagaacacgtggcagcagggagaaacctt

cacctgtgtggtgatgcacgaggccctgcacaaccactacacccagaaatccatcacccagtcttcgggtaaatagtaatctaga

Llama IgG2
Dqepktpkpqpqpqpqpnptteskcpkcpapellggpsvfifppkpkdvlsisgrpevtcvvvdvgq

edpevsfhwyidgaevrtantrpkeeqfnstyrvvsvlpiqhqdwltgkefkckvnnkalpapiektiskakggqtrepqvytla

phreelakdtvsvtclvkgfyppdinvewqmgqpesegtyattppqldndgtyflysklsvgkntwqqgctftcvvmhealh

nhytqksitqssgk

Llama IgG3 Fe
tgatcaagcgcaccacagcgaagaccccagctccaagtgtcccaaatgcccaggccctgaactccttgga

gggcccacggtcttcatcttcccccgaaagccaaggacgtcctctccatcacccgaaaacctgaggtcacgtgcttgtggtggac

gtgggtaaagaagaccctgagatcgagttcaagctggtccgtggatgacacagaggtacacacggctgagacaaagccaaagg

aggaacagttcaacagcacgtaccgcgtggtcagcgtcctgcccatccagcaccaggactggctgacggggaaggaattcaagt

gcaaggtcaacaacaaagctctcccagcccccatcgagaggaccatctccaaggccaaagggcagacccgggagccgcaggt

gtacaccctggccccacaccgggaagagctggccaaggacaccgtgagcgtaacctgcctggtcaaaggcttcttcccagctga

catcaacgttgagtggcagaggaatgggcagccggagtcagagggcacctacgccaacacgccgccacagctggacaacgac

gggacctacttcctctacagcaaactctccgtgggaaagaacacgtggcagcagggagaagtcttcacctgtgtggtgatgcacg

aggctctacacaatcactccacccagaaatccatcacccagtcttcgggtaaatagtaatctagagggccc

Llama IgG3 Fc
dqahhsedpsskepkcpgpellggptvfifppkakdvlsitrkpevtclwwtwvkktlrsssswsvddt

evhtaetkpkeeqfhstyrvvsvlpiqhqdwltgkefkekvnnkalpapiertiskakggqtrepqvytlaphreelakdtvsvte

lvkgffpadinvewqrngqpesegtyantppqldndgtyflysklsvgkntwqqgevftcvvmhealhnhstqksitqssgk

HuIgG1 wild type hinge
gatcaggagcccaaatcttgtgacaaaactcacacatgcccaccgtgcccagca

HuIgG1 wild type hinge
dqepkscdkthtcppepa

HuIgG1 H2, wild type hinge with leu at second position (results from BglI site)
gatctggagcccaaatcttgtgacaaaactcacccatgcccaccgtgcccagca

HuIgG1 H2, wild type hinge with leu at second position.
dlepksedkthtcppcpa

NT

-continued

HuIgG1 wild type CH2
cctgaactcctgggggaccgtcagtcttcctcttccccccaaaacccaaggacaccctcatgatctcccgg

acccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgaggtcaagttcaactggtacgtggacggcgtggag

gtgcataatgccaagacaaagccgcgggaggagcagtacaacagcacgtaccgtgtggtcagcgtcctcaccgtcctgcaccag

gactggctgaatggcaaggagtacaagtgcaaggtctccaacaaagccctcccagcccccatcgagaaaaccatctccaaagcc

aaa

HuIgG1 wild typc CH2 AA
pellggpsvflfppkpkdtlmisrtpevtcvvvdvshedpevkthwyvdgvevhnaktkpreeqynst

yrvvsvltvlhqdwlngkeykckvsnkalpapiektiskak

NT HuIgG1 wild type CH3
gggcagccccgagaaccacaggtgtacaccctgcccccatcccgggaggagatgaccaagaaccaggt

cagcctgacctgcctggtcaaaggcttctatcccagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaacta

caagaccacgcctcccgtgctggactccgaeggctccttettcctctatagcaagctcaccgtggacaagagcaggtggcagcag

gggaacgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacgcagaagagcctctccctgtccccgggtaaatg

a

AA HuIgG1 wild type CH3
gqprepqvytlppsreemtknqvsltclvkgfypsdiavewesngqpennykttppvldsdgsfflysk

ltvdksrwqqgnvfscsvmhealhnhytqkslslspgk

NT HuIgG1 mutated hinge (C—C—C—→S—S—S)
gatcaggagcccaaatcttctgacaaaactcacacatCcccaccgtccccagca

AA HuIgG1 mutated hinge (C—C—C—→S—S—S)
dqepkssdkthtsppspa

Mutant hinge, but wild type CH2 and CH3—reads from the hinge+Ig tail, HIgG1MTH WTCH2CH3:
tgatcaccccaaatcttctgacaaaactcacacatctccaccgtcctcagcacctgaactcctgggtggaccg

tcagtcttcctcttccccccaaaacccaaggacaccctcatgatctcccggacccctgaggtcacatgcgtggtggtggacgtgag

ccacgaagaccctgaggtcaagttcaactggtacgtggacggegtggaggtgcataatgccaagacaaagccgcgggaggagc

agtacaacagcacgtaccgtgtggtcagcgtcctcaccgtcctgcaccaggactggctgaatggcaaggagtacaagtgcaaggt

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ggagtgggagagcaatgggcagccggagaacaactacaagaccacgcctcccgtgctggactccgacggctcctcttcctcta

cagcaagctcaccgtggacaagagcaggtggcagcaggggaacgtcttctcatgctccgtgatgcatgaggctctgcacaacca

ctacacgcagaagagcctctccctgtctccgggtaaatgataatctaga

Protein sequence: Mutant hinge, but wild type CH2 and CH3
dhpkssdkthtsppssapellggpsvflfppkpkdtlmisrtpevtcvvvdvshedpevkthwyvdgv

evhnaktkpreeqynstyrvvsvltvlhqdwlngkeykekvsnkalpapiektiskakgqprepqvytlppsrdeltknqvslt

clvkgfypsdiavewesngqpennykttppvldsdgsfflyskltvdksrwqqgflvfsesvmhealhflhytqkslslspgk

LLG1-5'bgl 35 mer Llama IgG1 5'
5'-gtt gtt gat caa gaa cca cat gga gga tgc acg tg-3'

LLG2-5'bgl 32 mer, Llama IgG2-5'
5'-gtt gtt gat caa gaa ccc aag aca cca aaa cc-3'

LLG3-5'bgl 33 mer, Llama IgG3-5'
5'-gtt gtt gat caa gcg cac cac agc gaa gac ccc-3'

LLseqsense 19 mer, llama sequencing primer
5'-ctg aga tcg agt tca gct g-3'

LLseqAS 19 mer

-continued

5'-cct cct ttg gct ttg tct c-3'

NT
2H7 scFv llama IgG1
aagcttgccgccatggattttcaagtgcagattttcagcttcctgctaatcagtgcttcagtcataattgccagag

gacaaattgttctctcccagtctccagcaatcctgtctgcatctccaggggagaaggtcacaatgacttgcagggccagctcaagtg

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AA 2117 scFv llama IgGI
mdfqvqifsfllisasvllargqivlsqspailsaspgekvtmtcrasssvsymhwyqqkpgsspkpwiy

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NT 2117 scFv llama IgG2
aagcttgccgccatggattttcaagtgcagattttcagcttcctgctaatcagtgcttcagtcataattgccagag

gacaaattgttctctcccagtctccagcaatcctgtctgcatctccaggggagaaggtcacaatgacttgcagggccagctcaagtg

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AA
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mdfqvqifsfllisasviiargqivlsqspailsaspgekvtmtcrasssvsymhwyqqkpgsspkpwiy

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NT
2H7 scFv llama IgG3
aagcttgccgccatggattttcaagtgcagattttcagcttcctgctaatcagtgcttcagtcataattgccagag

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AA
2H7 scFv llama IgG3
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ytlaphreelakdtvsvtclvkgffpadinvewqrngqpesegtyantppqldndgtyflysklsvgkntwqqgevftcvvm

healhnhstqksitqssgk

2H7+Completely WT IgG tail:
2H7 scFv WTH WTCH2CH3
Nucleotide sequence:

aagcttgccgccatggattttcaagtgcagattttcagcttcctgctaatcagtgcttcagtcataattgccagag

gacaaattgttctctcccagtctccagcaatcctgtctgcatctccaggggagaaggtcacaatgacttgcagggccagctcaagtg

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2H7+Completely WT IgG tail:
2H7 scFv WTH WTCH2CH3
Protein sequence

mdfqvqifsfllisasviiargqivlsqspailsaspgekvtmtcrasssvsymhwyqqkpgsspkpwiy

apsnlasgvparfsgsgsgtsysltisrveaedaatyycqqwsfrippt fgagtklelkdgggsggggsggggssqaylqqsgae

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pqvytlppsrdeltknqvsltclvkgfypsdiavewesngqpennykttppvldsdgsfflyskltvdksrwqqgnvfscsvm

healhinhytqkslslspgk

NT
CD80 transmembrane domain and cytoplasmic tail (+restriction sites)
gcggatccttcgaacctgctcccatcctgggccattaccttaatctcagtaaatggaatttttgtgatatgctgcc

tgacctactgctttgccccaagatgcagagagagaaggaggaatgagagattgagaagggaaagtgtacgccctgtataaatcga

t

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AA
CD80 transmembrane domain and cytoplasmic tall
adpsnllpswaitlisvngifviccltycfaprcrerrrnerlrresvrpv

NT
40.2.220 VL (anti-human CD40 scFv #1--VL)
aagcttatggattttcaagtgcagattttcagcttcctgctaatcagtgcttcagtcataatgtccagaggagtcg

acattgttctgactcagtctccagccaccctgtctgtgactccaggagatagagtctctctttcctgcagggccagccagagtattagc

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tggtcacagctttccgtggacgttcggtggaggcaccaagctggaaatcaaacgg

AA
40.2.220 VL (anti-human CD40 scFv #1--VL)
mdfqvqifsfllisasvimsrgvdivltqspatlsvtpgdrvslscrasqsisdylhwyqqkshesprlliky

ashsisgipsrfsgsgsgsdftlsinsvepedvgiyycqhghsfpwtfgggtkleikr

NT
40.2.220 VH (for anti-human CD40 scFv #1--VH)
cagatccagttggtgcaatctggacctgagctgaagaagcctggagagacagtcaggatctcctgcaaggc

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gggccaagggacactggtcactgtctctgatca

AA
40.2.220 VH (for anti-human CD40 scFv #1--VH)
qiqlvqsgpelkkpgctvrisckasgyafttgmqwvqempgkglkwigwintplwsakicrrlqgrfa

fsletsantaylqisnlkdedtatyfcvrsgngnydlayfaywgqgtlvtvs

NT
40.2.220 scFv (anti-human CD40 scFv #1)
aagcttatggattttcaagtgcagattttcagcttcctgctaatcagtgcttcagtcataatgtccagaggagtcg

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AA
40.2.220 scFv (anti-human CD40 scFv HI
mdfqvqifsfllisasvimsrgvdivltqspatlsvtpgdrvslscrasqsisdylhwyqqkshesprlliky

ashsisgipsrfsgsgsgsdftlsinsvepedvgiyycqhghsfpfgggtkleikrggggsggggsggggsqiqlvqsgpel

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vrsgngnydlayfaywgqgtlvtvs

NT
2e12 VL (with L6 VK leader peptide)
atggattttcaagtgcagattttcagcttcctgctaatcagtgcttcagtcataatgtccagaggagtcgacattg

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tgctcacccaatctccagcttctttggctgtgtctctaggtcagagagccaccatctcctgcagagccagtgaaagtgttgaatattat

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AA
2e12 VL (with L6 VK leader peptide)
mdfqvqifsfllisasvimsrgvdivltqspaslavslgqratiscrasesveyyvtslmqwyqqkpgqp

pkllisaasnvesgvparfsgsgsgtdfslnihpveeddiamyfcqqsrkvpwtfgggtkleikr

NT
2e12 VH (no leader peptide)
caggtgcagctgaaggagtcaggacctggcctggtggcgccctcacagagcctgtccatcacatgcaccg

tctcagggttctcattaaccggctatggtgtaaactgggttcgccagcctccaggaaagggtctggagtggctgggaatgatatggg

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ggtcaaggaacctcagtcaccgtctcctca(gatctg)

AA
2e12 VH
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hIgGWT3xba (3' oligo for above mutation introduction)
gtt gtt tct aga tca ttt acc cgg aga cag gga gag gct ctt ctg cgt gta g

Vhser11: (oligo for Leu to ser at vH11)
gga ggt ggg agc tct cag gct tat cta cag cag tct ggg gct gag tcg gtg agg cc

huIgG1-3' (3' oligo to amplify IgG1C regions, 3' end of CH3)
gtc tct aga cta tca ttt acc cgg aga cag

hulgA/Gchim5 (oligo for pcr#1)
cca tct ccc tca act cca cct acc cca tct ccc tca tgc gca cct gaa ctc ctg

huIgAhg-5' (oligo for pcr#2)
gtt gtt gat cag cca gtt ccc tca act cca cct acc cca tct ccc caa ct

huIgA3'
gtt gtt tct aga tta tca gta gca ggt gcc gtc cac ctc cgc cat gac aac

2H7 scFv JgAH LGG WT CH2CH3, 2H7 scFv with IgA hinge and WT
CH2 and CH3
aagcttgccgccatggattttcaagtgcagattttcagcttcctgctaatcagtgcttcagtcataattgccagag

gacaaattgttctctcccagtctccagcaatcctgtctgcatctccaggggagaaggtcacaatgacttgcagggccagctcaagtg

taagttacatgcactggtaccagcagaagccaggatcctcccccaaaccctggatttatgccccatccaacctggcttctggagtcc

ctgctcgcttcagtggcagtgggtctgggacctcttactctctcacaatcagcagagtggaggctgaagatgctgccacttattactg

ccagcagtggagttttaacccacccacgttcggtgctgggaccaagctggagctgaaagatggcggtggctcgggcggtggtgg

atctggaggaggtgggagctctcaggcttatctacagcagtctggggctgagctggtgaggcctggggcctcagtgaagatgtcct

gcaaggcttctggctacacatttaccagttacaatatgcactgggtaaagcagacacctagacagggcctggaatggattggagct

-continued

atttatccaggaaatggtgatacttcctacaatcagaagttcaagggcaaggccacactgactgtagacaaatcctccagcacagcc

tacatgcagctcagcagcctgacatctgaagactctgcggtctatttctgtgcaagagtggtgtactatagtaactcttactggtacttc

gatgtctggggcacagggaccacggtcaccgtctctgatcagccagttccctcaactccacctaccccatctccctcaactccacct

accccatctccctcatgcgcacctgaactcctgggggggaccgtcagtcttcctcttccccccaaaacccaaggacaccctcatgatc

tcccggacccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgaggtcaagttcaactggtacgtggacggc

gtggaggtgcataatgccaagacaaagccgcgggaggagcagtacaacagcacgtaccgtgtggtcagcgtcctcaccgtcctg

caccaggactggctgaatggcaaggagtacaagtgcaaggtctccaacaaagccctcccagcccccatcgagaaaacaatctcc

aaagccaaagggcagccccgagaaccacaggtgtacaccctgcccccatcccgggatgagctgaccaagaaccaggtcagcct

gacctgcctggtcaaaggcttctatcccagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaactacaaga

ccacgcctcccgtgctggactccgacggctccttcttcctctacagcaagctcaccgtggacaagagcaggtggcagcagggga

acgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacgcagaagagcctctccctgtctccgggtaaatgatctag

a

2H7 scFv IgAH IGG WT CH2CH3 protein sequence
mdfqvqifsfllisasviiargqivlsqspailsaspgekvtmterasssvsymhwyqqkpgsspkpwiy

apsnlasgvparfsgsgsgtsysltisrveaedaatyycqqwsfnpptfgagtklelkdgggsggggsggggssqaylqqsgae

lvrpgasvkmsckasgytftsynmhwvkqtprqglewigaiypgngdtsynqkfkgkatltvdkssstaymqlssltsedsa

vyfcarvvyysnsywyfdvwgtgttvtvsdqpvpstpptpspstpptpspscapellggpsvflfppkpkdtlmisrtpevtcv

vvdvshedpevkthwyvdgvevhnaktkpreeqynstyrvvsvltvlhqdwlngkeykckvsnkalpapiektiskakgq

prepqvytlppsrdeltknqvsltclvkgfypsdiavewesngqpennykttppvldsdgsfflyskltvdksrwqqgnvfscs

vmhealhnhytqkslslspgk

NT
2H7 scFv IgAH IgACH2CH3 (2H7 scFv IgAhinge and IgA CH2 and CH3)
aagcttgccgccatggattttcaagtgcagattttcagcttcctgctaatcagtgcttcagtcataattgccagag

gacaaattgttctctcccagtctccagcaatcctgtctgcatctccaggggagaaggtcacaatgacttgcagggccagctcaagtg

taagttacatgcactggtaccagcagaagccaggatcctcccccaaaccctggatttatgccccatccaacctggcttctggagtcc

ctgctcgcttcagtggcagtgggtctgggacctcttactctctcacaatcagcagagtggaggctgaagatgctgccacttattactg

ccagcagtggagttttaacccacccacgttcggtgctgggaccaagctggagctgaaagatggcggtggctcgggcggtggtgg

atctggaggaggtgggagctctcaggcttatctacagcagtctggggctgagctggtgaggcctggggcctcagtgaagatgtcct

gcaaggcttctggctacacatttaccagttacaatatgcactgggtaaagcagacacctagacagggcctggaatggattggagct

atttatccaggaaatggtgatacttcctacaatcagaagttcaagggcaaggccacactgactgtagacaaatcctccagcacagcc

tacatgcagctcagcagcctgacatctgaagactctgcggtctatttctgtgcaagagtggtgtactatagtaactcttactggtacttc

gatgtctggggcacagggaccacggtcaccgtctcttctgatcagccagttccctcaactccacctaccccatctccctcaactcca

cctaccccatctccctcatgctgccaccccgactgtcactgcaccgaccggccctcgaggacctgctcttaggttcagaagcgat

cctcacgtgcacactgaccggcctgagagatgcctcaggtgtcaccttcacctggacgccctcaagtgggaagagcgctgttcaa

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ccttcacttgcactgctgcctaccccgagtccaagaccccgctaaccgccaccctctcaaaatccggaaacacattccggcccgag

gtccacctgctgccgccgccgtcggaggagctggccctgaacgagctggtgacgctgacgtgcctggcacgtggcttcagcccc

aaggatgtgctggttcgctggctgcaggggtcacaggagctgccccgcgagaagtacctgacttgggcatcccggcaggagccc

agccagggcaccaccaccttcgctgtgaccagcatactgcgcgtggcagccgaggactggaagaaggggacaccttctcctg

catggtgggccacgaggccctgccgctggccttcacacagaagaccatcgaccgcttggcgggtaaacccacccatgtcaatgt

-continued

gtctgttgtcatggcggaggtggacggcacctgctactgataatctaga

AA
2H7 scFv IgAH IgACH2CH3 (2H7 scFv IgA hinge and IgA CH2 and CH3)

mdfqvqifsfllisasviiargqivlsqspailsaspgekvtmtcrasssvsymhwyqqkpgsspkpwiy

apsnlasgvparfsgsgsgtsysltisrveaedaatyycqqwsfnpptfgagtklelkdgggsggggsggggssqaylqqsgae

lvrpgasvkmsckasgytftsynmhwvkqtprqglewigaiypgngdtsynqkfkgkatltvdkssstaymqlssltsedsa

vyfcarvvyysnsywyfdvwgtgttvtvssdqpvpstpptpspstpptpspscchprlslhrpaledlllgseailtctltglrdas

gvtftwtpssgksavqgppdrdlcgcysvssvlpgcaepwnhgktftctaaypesktpltatlsksgflttitpevhllpppseelal

nelvtltclargfspkdvlvrwlqgsqelprekyltwasrqepsqgtttfavtsilrvaaedwkkgdtfscmvghealplaftqkti

drlagkpthvnvsvvmaevdgtcy

IgA hinge-CH2-CH3 (Human IgA tail, full length)
tgatcagccagttccctcaactccacctaccccatctccctcaactccacctaccccatctccctcatgctgcc

acccccgactgtcactgcaccgaccggccctcgaggacctgctcttaggttcagaagcgatcctcacgtgcacactgaccggcct

gagagatgcctcaggtgtcaccttcacctggacgccctcaagtgggaagagcgctgttcaaggaccacctgaccgtgacctctgt

ggctgctacagcgtgtccagtgtcctgccgggctgtgccgagccatggaaccatgggaagaccttcacttgcactgctgcctaccc

cgagtccaagaccccgctaaccgccaccctctcaaaatccggaaacacattccggcccgaggtccacctgctgccgccgccgtc

ggaggagctggccctgaacgagctggtgacgctgacgtgcctggcacgtggcttcagccccaaggatgtgctggttcgctggct

gcaggggtcacaggagctgccccgcgagaagtacctgacttgggcatcccggcaggagcccagccagggcaccaccaccttc

gctgtgaccagcatactgcgcgtggcagccgaggactggaagaagggggacaccttctcctgcatggtgggccacgaggccct

gccgctggccttcacacagaagaccatcgaccgcttggcgggtaaacccacccatgtcaatgtgtctgttgtcatggcggaggtgg

acggcacctgctactgataatctaga

IgA hinge-CH2-CH3 protein sequence, (Human IgA tail, full length)
Dqpvpstpptpspstpptpspscchprlslhrpaledlllgseailtctltglrdasgvtftwtpssgksavqg

ppdrdlcgeysvssvlpgcaepwnhgktftctaaypesktpltatlsksgntfrpeVhllpppseelalnelvtltelargfspkdvl

vrwlqgsqelprekyltwasrqepsqgtttfavtsilrvaaedwkkgdtfsemvghealplaftqktidrlagkpthvnvsvvm

aevdgtcy

Human J Chain:
agatctcaagaagatgaaaggattgttcttgttgacaacaaatgtaagtgtgcccggattacttccaggatcat

ccgttcttcgaagatcctaatgaggacattgtggagagaaacatccgaattattgttcctctgaacaacagggagaatatctctgatc

ccacctcaccattgagaaccagatttgtgtaccatttgtctgacctcagctgtaaaaaatgtgatcctacagaagtggagctggataat

cagatagttactgctacccagagcaatatctgtgatgaagacagtgctacagagacctgctacacttatgacagaaacaagtgctac

acagctgtggtcccactcgtatatggtggtgagaccaaaatggtggaaacagccttaaccccagatgcctgctatcctgactaatcta

ga

Human J Chain polypeptide
rsqederivlvdnkckcaritsriirssedpnediverniriivplnnrenisdptsplrtrfvyhlsdlsckkc

dptevеldnqivtatqsnicdedsatctcytydrnkcytavvplvyggctkmvetaltpdacyp

HUICH5nl (J chain 5' primer)
gtt gtt aga tct caa gaa gat gaa agg att gtt ctt

HUICH3 (J chain 3' primer-antisense)
gtt gtt tct aga tta gtc agg ata gca ggc ate tgg

4 carboxy terminal amino acids deleted from IgA CH3
GTCY

-continued

IgAH LgAT4 Human IgA tail, truncated (3T1)-(missing last 4 amino acids from carboxy terminus)

tgatcagccagttccctcaactccacctaccccatctccctcaactccacctaccccatctccctcatgctgcc

acccccgactgtcactgcaccgaccggccctcgaggacctgctcttaggttcagaagcgatcctcacgtgcacactgaccggcct

gagagatgcctcaggtgtcaccttcacctggacgccctcaagtgggaagagcgctgttcaaggaccacctgaccgtgacctctgt

ggctgctacagcgtgtccagtgtcctgccgggctgtgccgagccatggaaccatgggaagaccttcacttgcactgctgcctaccc

cgagtccaagaccccgctaaccgccaccctctcaaaatccggaaacacattccggcccgaggtccacctgctgccgccgccgtc

ggaggagctggccctgaacgagctggtgacgctgacgtgcctggcacgtggcttcagccccaaggatgtgctggttcgctggct

gcaggggtcacaggagctgccccgcgagaagtacctgacttgggcatcccggcaggagcccagccagggcaccaccaccttc

gctgtgaccagcatactgcgcgtggcagccgaggactggaagaagggggacaccttctcctgcatggtgggccacgaggccct

gccgctggccttcccacagaagaccatcgaccgcttggcgggtaaacccacccatgtcaatgtgtctgttgtcatggcggaggtgg

actgataatctaga

IgAH IgAT4 protein sequence:

Dujvpstpptpspstpptpspscchprlslhrpaledlllgseailtctltglrdasgvtftwtpssgksavqg

ppdrdlcgcysvssvlpgcaepwnhgktftctaaypesktpltatlsksgntfrpeVhllpppseelalnelvtltelargfspkdvl

vrwlqgsqelprekyltwasrqepsqgtttfavtsilrvaaedwkkgdtfscmvghealplaftqktidrlagkpthvnvsvvm

aevd

HUIGA3T1 (Oligo 3': to delete 4 amino acids at carboxy end of IgA CH3)

gtt gtt tct aga tta tca gtc cac ctc cgc cat gac aac aga cac

HUIGA3T2: (oligo to delete 14 aa at end of IgA -T4)

gtt gtt tct aga tta tca ttt acc cgc caa gcg gtc gat ggt ett

NT

2H7scFvIgAHIgAT4

(2H7 scFv IgA 3T1 construct)--truncates the CH3 domain at the 3'end

aagcttgccgccatggattttcaagtgcagattttcagcttcctgctaatcagtgcttcagtcataattgccagag

gacaaattgttctctcccagtctccagcaatcctgtctgcatctccaggggagaaggtcacaatgacttgcagggccagctcaagtg

taagttacatgcactggtaccagcagaagccaggatcctcccccaaaccctggatttatgccccatccaacctggcttctggagtcc

ctgctcgcttcagtggcagtgggtctgggacctcttactctctcacaatcagcagagtggaggctgaagatgctgccacttattactg

ccagcagtggagttttaacccacccacgttcggtgctgggaccaagctggagctgaaagatggcggtggctcgggcggtggtgg

atctggaggaggtgggagctctcaggcttatctacagcagtctggggctgagctggtgaggcctggggcctcagtgaagatgtcct

gcaaggcttctggctacacatttaccagttacaatatgcactgggtaaagcagacacctagacagggcctggaatggattggagct

atttatccaggaaatggtgatacttcctacaatcagaagttcaagggcaaggccacactgactgtagacaaatcctccagcacagcc

tacatgcagctcagcagcctgacatctgaagactctgcggtctatttctgtgcaagagtggtgtactatagtaactcttactggtacttc

gatgtctggggcacagggaccacggtcaccgtctcttctgatcagccagttccctcaactccacctaccccatctccctcaactcca

cctaccccatctccctcatgctgccacccccgactgtcactgcaccgaccggccctcgaggacctgctcttaggttcagaagcgat

cctcacgtgcacactgaccggcctgagagatgcctcaggtgtcaccttcacctggacgccctcaagtgggaagagcgctgttcaa

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gtccacctgctgccgccgccgtcggaggagctggccctgaacgagctggtgacgctgacgtgcctggcacgtggcttcagcccc

aaggatgtgctggttcgctggctgcaggggtcacaggagctgccccgcgagaagtacctgacttgggcatcccggcaggagccc

agccagggcaccaccaccttcgctgtgaccagcatactgcgcgtggcagccgaggactggaagaagggggacaccttctcctg

catggtgggccacgaggccctgccgctggccttcacacagaagaccatcgaccgcttggcgggtaaacccacccatgtcaatgt

gtctgttgtcatggcggaggtggactgataatctaga

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AA
2H7 scFv IgAH-T4
mdfqvqifsfllisasviiargqivlsqspailsaspgekvtmtcrasssvsymhwyqqkpgsspkpwiy

apsnlasgvparfsgsgsgtsysltisrveaedaatyyeqqwsfnpptfgagtklelkdgggsggggsggggssqaylqqsgae

lvrpgasvkmsekasgytftsynmhwvkqtprqglewigaiypgngdtsynqkfkgkatltvdkssstaymqlssltsedsa

vyfearvvyysnsywyfdvwgtgttvtvssdqpvpstpptpspstpptpspscchprlslhrpaledlllgseailtctltglrdas

gvtftwtpssgksavqgppdrdlcgcysvssvlpgcacpwnhgktftctaaypesktpltatlsksgntfrpeVhllpppseelal

nelvtltclargfspkdvlvrwlqgsqelprekyltwasrqepsqgtttfavtsilrvaaedwkkgdtfscmvghealplaftqkti

drlagkpthvnvsvvmaevd

14 amino acids deleted from IgAH-T4 (so that total of 18 amino acids deleted from wild type IgA CH3
PTHVNVSVVMAEVD

IgAH IgA-T 8 (Human IgA Tail truncated, 3T2)
Tgatcagccagttccctcaactccacctaccccatctccctcaactccacctaccccatctccctcatgctgcc

acccccgactgtcactgcaccgaccggccctcgaggacctgctcttaggttcagaagcgatcctcacgtgcacactgaccggcct

gagagatgcctcaggtgtcaccttcacctggacgccctcaagtgggaagagcgctgttcaaggaccacctgaccgtgacctctgt

ggctgctacagcgtgtccagtgtcctgccgggctgtgccgagccatggaaccatgggaagaccttcacttgcactgctgcctaccc

cgagtccaagaccccgctaaccgccaccctctcaaaatccggaaacacattccggcccgaggtccacctgctgccgccgccgtc

ggaggagctggccctgaacgagctggtgacgctgacgtgcctggcacgtggcttcagccccaaggatgtgctggttcgctggct

gcaggggtcacaggagctgccccgcgagaagtacctgacttgggcatcccggcaggagcccagccagggcaccaccaccttc

gctgtgaccagcatactgcgcgtggcagccgaggactggaagaagggggacaccttctcctgcatggtgggccacgaggccct

gccgctggccttcacacagaagaccatcgaccgcttggcgggtaaa

IgAH LgA-T18 protein sequence:
dqpvpstpptpspstpptpspscchprlslhrpaledlllgseailtcthglrdasgvtftwtpssgksavqg

ppdrdlcgcysvssvlpgcacpwnhgktftctaaypesktpltatlsksgntfrpeVhllpppseelalnelvtltclargfspkdvl

vrwlqgsqelprekyltwasrqepsqgtttfavtsilrvaaedwkkgdtfscmvghealplaftqktidrlagk

NT
2H7 scFv IgAH IgAT18: (Human IgA Tail truncated, 3T2.)
aagcttgccgccatggattttcaagtgcagattttcagcttcctgctaatcagtgcttcagtcataattgccagag

gacaaattgttctctcccagtctccagcaatcctgtctgcatctccaggggagaaggtcacaatgacttgcagggccagctcaagtg

taagttacatgcactggtaccagcagaagccaggatcctcccccaaaccctggatttatgccccatccaacctggcttctggagtcc

ctgctcgcttcagtggcagtgggtctgggacctcttactctctcacaatcagcagagtggaggctgaagatgctgccacttattactg

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tacatgcagctcagcagcctgacatctgaagactctgcggtctatttctgtgcaagagtggtgtactatagtaactcttactggtacttc

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cctaccccatctccctcatgctgccacccccgactgtcactgcaccgaccggccctcgaggacctgctcttaggttcagaagcgat

cctcacgtgcacactgaccggcctgagagatgcctcaggtgtcaccttcacctggacgccctcaagtgggaagagcgctgttcaa

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catggtgggccacgaggccctgccgctggccttcacacagaagaccatcgaccgcttggcgggtaaa

AA:
2H7 scFv IgAH IgAT18:
mdfqvqifsflisasviiargqivlsqspailsaspgekvtmtcrasssvsymhwyqqkpgsspkpwiy

apsnlasgvparfsgsgsgtsysltisrveaedaatyycqqwsfnpptfgagtklelkdgggsggggsggggssqaylqqsgae

lvrpgasvkmsckasgytftsynmhwvkqtprqglewigaiypgngdtsynqkfkgkatltvdkssstaymqlssltsedsa

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gvtftwtpssgksavqgppdrdlcgcysvssvlpgcacpwnhgktftctaaypesktpltatlsksgntfrpevhllpppseelal

nelvtltclargfspkdvlvrwlqgsqelprekyltwasrqepsqgtttfavtsilrvaaedwkkgdtfscrnvghealplaftqkti

drlagk

CTLA-4 IgG WTH WTCH2CH3 (Human-oncoMLp-CTLA4EC-hIgGWT)
Nucleotide sequence:
gcaacctacatgatggggaatgagttgaccttcctagatgattccatctgcacgggcacctccagtggaaatc

aagtgaacctcactatccaaggactgagggccatggacacgggactctacatctgcaaggtggagctcatgtacccaccgccata

ctacctgggcataggcaacggaacccagatttatgtaattgatccagaaccgtgcccagattctgatcaacccaaatcttgtgacaaa

actcacacatgcccaccgtgcccagcacctgaactcctggggggaccgtcagtcttcctcttccccccaaaacccaaggacaccct

catgatctcccggacccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgaggtcaagttcaactggtacgtg

gacggcgtggaggtgcataatgccaagacaaagccgcgggaggagcagtacaacagcacgtaccgtgtggtcagcgtcctcac

cgtcctgcaccaggactggctgaatggcaaggagtacaagtgcaaggtctccaacaaagccctcccagcccccatcgagaaaac

aatctccaaagccaaagggcagccccgagaaccacaggtgtacaccctgcccccatcccgggatgagctgaccaagaaccagg

tcagcctgacctgcctggtcaaaggcttctatcccagcgacatcgccgtggagtgggagagcaatgggcagccggagaacaact

acaagaccacgcctcccgtgctggactccgacggctccttcttcctctacagcaagctcaccgtggacaagagcaggtggcagca

ggggaacgtcttctcatgctccgtgatgcatgaggctctgcacaaccactacacgcagaagagcctctccctgtctccgggtaaatg

a

CTLA-4 IgG WTH WTCH2CH3 protein sequence:
mgvlltqrtllslvlallfpsmasmamhvaqavvlassrgtasfvceyaspgkatevrvtvlrqadsqvt

evcaatymmgnehflddsictgtssgnqvnhiqglramdtglyickvelmypppyylgigngtqiyvidpepcpdsdqpks

cdkthtcppcpapellggpsvflfppkpkdtlmisrtpevtcvvvdvshedpevkfnwyvdgvevhnaktkpreeqynstyr

vvsvhvlhqdwlngkeykckvsnkalpapiektiskakgqprepqvytlppsrdehknqvsltclvkgfypsdiavewesn

gqpennykttppvldsdgsfflyskltvdksrwqqgnvfscsvmhealhnhytqkslslspgk

Human OncoM leader peptide+CTLA4 EC (BclI)
Atgggggtactgctcacacagaggacgctgctcagtctggtccttgcactcctgtttccaagcatggcgagc

atggcaatgcacgtggcccagcctgctgtggtactggccagcagccgaggcatcgccagctttgtgtgtgagtatgcatctccagg

caaagccactgaggtccgggtgacagtgcttcggcaggctgacagccaggtgactgaagtctgtgcggcaacctacatgatggg

gaatgagttgaccttcctagatgattccatctgcacgggcacctccagtggaaatcaagtgaacctcactatccaaggactgagggc

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atgtaattgatccagaaccgtgcccagattctgatcaa

Human OncoM leader peptide+CTLA4 EC peptide sequence:
mgvlltqrtllslvlallfpsmasmamhvaqpavvlassrgtasfvceyaspgkatevrvtvlrqadsqvt

evcaatymmgneltflddsictgtssgnqvnltiqglramdtglyickvelmypppyylgigngtqiyvidpepcpdsdq

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Human OncoM leader peptide nucleotide
atgggggtactgctcacacagaggacgctgctcagtctggtccttgcactcctgtttccaagcatggcgagc

atg

Human OncoM leader peptide sequence:
Mgvlltqrtllslvlallfpsm

NT
Human CTLA4 EC (no Lp)
Gcaatgcacgtggcccagcctgctgtggtactggccagcagccgaggcatcgccagctttgtgtgtgagta

tgcatctccaggcaaagccactgaggtccgggtgacagtgcttcggcaggctgacagccaggtgactgaagtctgtgcggcaac

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cggaacccagatttatgtaattgatccagaaccgtgcccagattct

AA
Human CTLA4 EC (no Lp)
Amhvaqpavvlassrgtasfvceyaspgkatevrvtvlrcladsqvtevcaatymtgfleltflddsictgts

sgnqvnltiqglramdtglyickvelmypppyylgigngtqiyvidpepcpds

NT
Human CTLA4 IgG MTH (SSS) MTCH2CH3
Atgggggtactgctcacacagaggacgctgctcagtctggtccttgcactcctgtttccaagcatggcgagc

atggcaatgcacgtggcccagcctgctgtggtactggccagcagccgaggcatcgccagctttgtgtgtgagtatgcatctccagg

caaagccactgaggtccgggtgacagtgcttcggcaggctgacagccaggtgactgaagtctgtgcggcaacctacatgatggg

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AA
Human CTLA4 IgG MTH (SSS) MTCH2CH3
Mgvlltqrtllslvlallfpsmasmamhvaqpavvlassrgtasfvceyaspgkatevrvtvlrqadsqvt

evcaatymmgneltflddsictgtssgnqvnltiqglramdtglyickvelmypppyylgigngtqiyvidpepcpdsdqpks

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gqpennykttppvldsdgsfflyskltvdksrwqqgnvfscsvmhealhnhytqkslslspgk

CTLA-4 IgAH IgACH2CH3 (Human-oncoMLp-CTLA4EC-IgA)
Nucleotide sequence:
atgggggtactgctcacacagaggacgctgctcagtctggtccttgcactcctgtttccaagcatggcgagc

atggcaatgcacgtggcccagcctgctgtggtactggccagcagccgaggcatcgccagctttgtgtgtgagtatgcatctccagg

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ggcggaggtggacggcacctgctactgataatctaga

CTLA-4 IgAH IgACH2CH3 protein sequence:
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evcaatymmgneltflddsictgtssgnqvnhiqglramdtglyickvelmypppyylgignqtqiyvidpepcpdsdqpv

pstpptpspstpptpspscchprlslhrpaledlllgseailtctltglrdasgvtftwipssgksavqgppdrdlcgcysvssvlpg

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wasrqepsqgtttfavtsilrvaaedwkkgdtfscmvghealplafiqktidrlagkpthvnvsvvmaevdgtcy

CTLA-4 LgAH IgA-T4 (Human-oncoMLp-CTLA4EC-IgA3T1)
Nucleotide sequence:
atgggggtactgctcacacagaggacgctgctcagtctggtccttgcactcctgtttccaagcatggcgagc

atggcaatgcacgtggcccagcctgctgtggtactggccagcagccgaggcatcgccagctttgtgtgtgagtatgcatctccagg

caaagccactgaggtccgggtgacagtgcttcggcaggctgacagccaggtgactgaagtctgtgcggcaacctacatgatggg

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ggcggaggtggactgataatctaga

CTLA-4 IgAH IgA-T4 protein sequence:
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NT
human IgG1 CH2 with 238 mutation pro→ser
cctgaactcctggggggatcgtcagtcttcctcttccccccaaaacccaaggacaccctcatgatctcccgga

cccctgaggtcacatgcgtggtggtggacgtgagccacgaagaccctgaggtcaagttcaactggtacgtggacggcgtggagg

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actggctgaatggcaaggagtacaagtgcaaggtctccaacaaagccctcccagcccccatcgagaaaaccatctccaaagcca

aag

AA
human IgGI CH2 with 238 mutation pro-→ser
pellggssvflfppkpkdtlmisrtpevtcvvvdvshedpevkfnwyvdgveVhnaktkpreeqynst

yrvvsvltvlhqdwlngkeykckvsnkalpapiektiskak

Amino acids surrounding Pro to Ser in CH2
PAPELLGGPS

Amino acids surrounding Pro to Ser in CH2
PAPELLGGSS

HIgE5Bcl
gtt gtt gat cac gtc tgc tcc agg gac ttc acc cc

hIgE3 stop
gtt gtt tct aga tta act ttt acc ggg att tac aga cac cgc tcg ctg g

hIgE3BB (leaves an open reading frame at end of gene to read into
transmembrane and cytoplasmic tail domain attached at either the BamUl or sfuI sites)
gtt gtt ttc gaa gga tcc gct tta ccg gga ttt aca gac acc gct cgc tgg

NT
human IgE Fe (CH2—CH3-CH4) ORF:
tgatcacgtctgctccagggacttcaccccgcccaccgtgaagatcttacagtcgtcctgcgacggcggcg

ggcacttccccccgaccatccagctcctgtgcctcgtctctgggtacaccccagggactatcaacatcacctggctggaggacgg

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atctgccgtgcagtccatgaggcagcgagcccctcacagaccgtccagcgagcggtgtctgtaaatcccggtaaagcggatcctt

cgaa

AA
human IgE Fe (CH2—CH3-CH4) ORE:
dhvesrdftpptvkilqssedgggghfpptiqllelvsgytpgtinitwledgqvmdvdlstasttqegelast

qseltlsqkhwlsdrtyteqvtyqghtfedstkkeadsnprgvsaylsrpspfdlfirksptitelvvdlapskgtvflltwsrasgk

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nfrnpedisvqwlhnevqlpdarhsttqprktkgsgffvfsrlevtraeweqkdefieraVhcaaspsqtvqravsvflpgkadp

s

IFhIgGwtBcl5
gtt gtt tga tca gga gcc caa atc ttg tga caa aac tca cac atg ccc acc gtg ccc agc
acc (63 mer)

-continued

hIgGWT3xba
gtt gtt tct aga tca ttt acc cgg aga cag gga gag gct ctt ctg cgt gta g

HulgGMHwC (sense, 5' primer for mutating wild type hinge ccc to
mutant sss
gtt gtt gat cag gag ccc aaa tct tct gac aaa act cac aca tct cca ccg tcc cca gca

cct gaa etc ctg ggt gga ccg tca gtc tic c

NT
1D8 vH
caggtgcagctgaaggaggcaggacctggcctggtgcaaccgacacagaccctgtccctcacatgcactg

tctctgggttctcattaaccagcgatggtgtacactggattcgacagcctccaggaaagggtctggaatggatgggaataatatattat

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aacagtctgcaaactgatgacacagccatgtattactgtgccagaatccactttgattactggggccaaggagtcatggtcacagtct

cctct

AA
1D8 VH (no leader)
qvqlkcagpglvqptqtlsltctvsgfsltsdgVhwirqppgkglewmgttyydggtdynsaiksrlsisr

dtsksqvflkinslqtddtamyycarihfdywgqgvmvtvss

NT
1Ds VL (no leader)
gacattgtgctcactcagtctccaacaaccatagctgcatctccaggggagaaggtcaccatcacctgccgt

gccagctccagtgtaagttacatgtactggtaccagcagaagtcaggcgcctcccctaaactctggatttatgacacatccaagctg

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ccacttattactgtcagcagtggagtagtactccgctcacgttcgggtctgggaccaagctggagatcaaacgg

AA
1D8 VL
divltqspttiaaspgekvtitcrasssvsymywyqqksgaspklwiydtsklasgvpnrfsgsgsgtsys

laintmetedaatyycqqwsstpltfgsgtkleikr

NT
1D8 scFv
aagcttatggattttcaagtgcagattttcagcttcctgctaatcagtgcttcagtcataatgtccagaggagtcg

acattgtgctcactcagtctccaacaaccatagctgcatctccaggggagaaggtcaccatcacctgccgtgccagctccagtgtaa

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AA
1D8 scFv
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dtsklasgvpnrfsgsgsgtsyslaintmetedaatyycqqwsstpltfgsgtkleikrggggsggggsggggsqvqlkcagpg

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rihfdywgqgvmvtvss

NT

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1D8 scFv IgG WTH WTCH2CH3
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NT
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AA
1D8 scFv IgG MTH MTCH2CH3-CD80
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5B9 VH no leader Peptide
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5' oligo:

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3' oligo:
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5' oligo:
Name IgGwT3
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CTT

3' oligo:
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5' oligo:
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Name MFADD3XC

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Sequence
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Murine FADD Nucleotide Sequence (full length, but without flanking -Ig
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Name: MCASP3-3
Sequence
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Name: mcasp8-5
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Name: mcasp8-3
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-continued

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[0362]

SEQUENCE LISTING

The patent application contains a lengthy "Sequence Listing" section. A copy of the "Sequence Listing" is available in electronic form from the USPTO web site (http://seqdata.uspto.gov/sequence.html?DocID=20030118592). An electronic copy of the "Sequence Listing" will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

What is claimed is:

1. A binding domain-immunoglobulin fusion protein, comprising:

(a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein said hinge region polypeptide is selected from the group consisting of (i) a wild-type human IgG1 immunoglobulin hinge region polypeptide, (ii) a mutated human IgG1 immunoglobulin hinge region polypeptide that is derived from a wild-type immunoglobulin hinge region polypeptide having three or more cysteine residues, wherein said mutated human IgG1 immunoglobulin hinge region polypeptide contains two cysteine residues and wherein a first cysteine of the wild-type hinge region is not mutated, (iii) a mutated human IgG1 immunoglobulin hinge region polypeptide that is derived from a wild-type immunoglobulin hinge region polypeptide having three or more cysteine residues, wherein said mutated human IgG1 immunoglobulin hinge region polypeptide contains no more than one cysteine residue, and (iv) a mutated human IgG1 immunoglobulin hinge region polypeptide that is derived from a wild-type immunoglobulin hinge region polypeptide having three or more cysteine residues, wherein said mutated human IgG1 immunoglobulin hinge region polypeptide contains no cysteine residues;

(b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide; and

(c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide,

wherein:

(1) the binding domain-immunoglobulin fusion protein is capable of at least one immunological activity selected from the group consisting of antibody dependent cell-mediated cytotoxicity and complement fixation, and

(2) the binding domain polypeptide is capable of specifically binding to an antigen.

2. The binding domain-immunoglobulin fusion protein of claim 1 wherein the immunoglobulin hinge region polypeptide is a mutated hinge region polypeptide and exhibits a reduced ability to dimerize, relative to a wild-type human immunoglobulin G hinge region polypeptide.

3. The binding domain-immunoglobulin fusion protein of claim 1 wherein the binding domain polypeptide comprises at least one immunoglobulin variable region polypeptide that is selected from the group consisting of an immunoglobulin light chain variable region polypeptide and an immunoglobulin heavy chain variable region polypeptide.

4. A binding domain-immunoglobulin fusion protein according to claim 3 which comprises an immunoglobulin heavy chain variable region polypeptide, wherein said heavy chain variable region polypeptide is a human immunoglobulin heavy chain variable region polypeptide comprising a mutation at an amino acid at a location corresponding to amino acid position 11 in SEQ ID NO:______.

5. A binding domain-immunoglobulin fusion protein according to claim 3 which comprises a polypeptide having a sequence selected from the group consisting of SEQ ID NO:______ and SEQ ID NO:______.

6. The binding domain-immunoglobulin fusion protein of claim 3 wherein the immunoglobulin variable region polypeptide is derived from a human immunoglobulin.

7. The binding domain-immunoglobulin fusion protein of claim 3 wherein the immunoglobulin variable region polypeptide comprises a humanized immunoglobulin polypeptide sequence.

8. The binding domain-immunoglobulin fusion protein of claim 3 wherein the immunoglobulin variable region polypeptide is derived from a murine immunoglobulin.

9. The binding domain-immunoglobulin fusion protein of claim 1 wherein the binding domain polypeptide comprises:

(a) at least one immunoglobulin light chain variable region polypeptide;

(b) at least one immunoglobulin heavy chain variable region polypeptide; and

(c) at least one linker polypeptide that is fused to the polypeptide of (a) and to the polypeptide of (b).

10. The binding domain-immunoglobulin fusion protein of claim 9 wherein the immunoglobulin light chain variable region and heavy chain variable region polypeptides are derived from human immunoglobulins.

11. The binding domain-immunoglobulin fusion protein of claim 9 wherein the linker polypeptide comprises at least one polypeptide having as an amino acid sequence Gly-Gly-Gly-Gly-Ser [SEQ ID NO:______].

12. The binding domain-immunoglobulin fusion protein of claim 9 wherein the linker polypeptide comprises at least three repeats of a polypeptide having as an amino acid sequence Gly-Gly-Gly-Gly-Ser [SEQ ID NO:______].

13. A binding domain-immunoglobulin fusion protein according to claim 9 wherein the linker comprises a glycosylation site.

14. The fusion protein of claim 13 wherein the glycosylation site is selected from the group consisting of an asparagine-linked glycosylation site, an O-linked glycosylation site, a C-mannosylation site, a glypiation site and a phosphoglycation site.

15. The binding domain-immunoglobulin fusion protein of claim 1 wherein at least one of the immunoglobulin heavy chain CH2 constant region polypeptide and the immunoglobulin heavy chain CH3 constant region polypeptide is derived from a human immunoglobulin heavy chain.

16. The binding domain-immunoglobulin fusion protein of claim 1 wherein the immunoglobulin heavy chain constant region CH2 and CH3 polypeptides are of an isotype selected from the group consisting of human IgG and human IgA.

17. The binding domain-immunoglobulin fusion protein of claim 1 wherein the antigen is selected from the group consisting of CD19, CD20, CD22, CD37, CD40, L6, CD2, CD28, CD30, CD40, CD50 (ICAM3), CD54 (ICAM1), CD80, CD86, B7-H1, CD134 (OX40), CD137 (41BB), CD152 (CTLA-4), CD153 (CD30 ligand), CD154 (CD40 ligand), ICOS, CD19, CD3, CD4, CD25, CD8, CD11b, CD14, CD25, CD56 and CD69.

18. The binding domain-immunoglobulin fusion protein of claim 1 wherein the binding domain polypeptide comprises a CD154 extracellular domain.

19. The binding domain-immunoglobulin fusion protein of claim 1 wherein the binding domain polypeptide comprises a CD154 extracellular domain and at least one immunoglobulin variable region polypeptide.

20. The binding domain-immunoglobulin fusion protein of claim 1 wherein the binding domain polypeptide comprises a CTLA-4 extracellular domain.

21. The binding domain-immunoglobulin fusion protein of claim 20 wherein at least one of the immunoglobulin heavy chain constant region polypeptides selected from the group consisting of a CH2 constant region polypeptide and a CH3 constant region polypeptide is a human IgG1 constant region polypeptide.

22. The binding domain-immunoglobulin fusion protein of claim 20 wherein at least one of the immunoglobulin heavy chain constant region polypeptides selected from the group consisting of a CH2 constant region polypeptide and a CH3 constant region polypeptide is a human IgA constant region polypeptide.

23. A binding domain-immunoglobulin fusion protein, comprising:

(a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide;

(b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide; and

(c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide,

wherein:

(1) the binding domain polypeptide comprises a CTLA-4 extracellular domain that is capable of specifically binding to at least one CTLA-4 ligand selected from the group consisting of CD80 and CD86,

(2) the immunoglobulin hinge region polypeptide comprises a polypeptide that is selected from the group consisting of a human IgA hinge region polypeptide and a human IgG1 hinge region polypeptide,

(3) the immunoglobulin heavy chain CH2 constant region polypeptide comprises a polypeptide that is selected from the group consisting of a human IgA heavy chain CH2 constant region polypeptide and a human IgG1 heavy chain CH2 constant region polypeptide,

(4) the immunoglobulin heavy chain CH3 constant region polypeptide comprises a polypeptide that is selected from the group consisting of a human IgA heavy chain CH3 constant region polypeptide and a human IgG1 heavy chain CH3 constant region polypeptide, and

(5) the binding domain-immunoglobulin fusion protein is capable of at least one immunological activity selected from the group consisting of antibody dependent cell-mediated cytotoxicity and complement fixation.

24. A binding domain-immunoglobulin fusion protein, comprising:

(a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein said hinge region polypeptide comprises a human IgE hinge region polypeptide;

(b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide, wherein said CH2 constant region polypeptide comprises a human IgE CH2 constant region polypeptide; and

(c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region

polypeptide, wherein said CH3 constant region polypeptide comprises a human IgE CH3 constant region polypeptide

wherein:

(1) the binding domain-immunoglobulin fusion protein is capable of at least one immunological activity selected from the group consisting of antibody dependent cell-mediated cytotoxicity and induction of an allergic response mechanism, and

(2) the binding domain polypeptide is capable of specifically binding to an antigen.

25. A binding domain-immunoglobulin fusion protein according to claim 24 that comprises a human IgE CH4 constant region polypeptide.

26. The binding domain-immunoglobulin fusion protein of claim 24 wherein the antigen is a tumor antigen.

27. A binding domain-immunoglobulin fusion protein, comprising:

(a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein the binding domain polypeptide is capable of specifically binding to at least one antigen that is present on an immune effector cell and wherein the hinge region polypeptide comprises a polypeptide selected from the group consisting of a human IgA hinge region polypeptide, a human IgG hinge region polypeptide, and a human IgE hinge region polypeptide;

(b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide, wherein said CH2 constant region polypeptide comprises a polypeptide selected from the group consisting of a human IgA CH2 constant region polypeptide, a human IgG CH2 constant region polypeptide, and a human IgE CH2 constant region polypeptide;

(c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein said CH3 constant region polypeptide comprises a polypeptide selected from the group consisting of a human IgA CH3 constant region polypeptide, a human IgG CH3 constant region polypeptide, and a human IgE CH3 constant region polypeptide; and

(d) a plasma membrane anchor domain polypeptide.

28. The binding domain-immunoglobulin fusion protein of claim 27 wherein the membrane anchor domain polypeptide comprises a transmembrane domain polypeptide.

29. The binding domain-immunoglobulin fusion protein of claim 27 wherein the membrane anchor domain polypeptide comprises a transmembrane domain polypeptide and a cytoplasmic tail polypeptide.

30. The binding domain-immunoglobulin fusion protein of claim 29 wherein the cytoplasmic tail polypeptide comprises an apoptosis signaling polypeptide sequence.

31. The binding domain-immunoglobulin fusion protein of claim 30 wherein the apoptosis signaling polypeptide sequence is derived from a receptor death domain polypeptide.

32. The binding domain-immunoglobulin fusion protein of claim 31 wherein the death domain polypeptide comprises a polypeptide selected from the group consisting of an ITIM domain, an ITAM domain, FADD, TRADD, RAIDD, CD95 (FAS/Apo-1), TNFR1 and DR5.

33. The binding domain-immunoglobulin fusion protein of claim 30 wherein the apoptosis signaling polypeptide sequence comprises a polypeptide sequence derived from a caspase polypeptide that is selected from the group consisting of caspase-3 and caspase-8.

34. The binding domain-immunoglobulin fusion protein of claim 27 wherein the plasma membrane anchor domain polypeptide comprises a glycosyl-phosphatidylinositol-linkage polypeptide sequence.

35. The binding domain-immunoglobulin fusion protein of claim 27 wherein the antigen that is present on an immune effector cell is selected from the group consisting of CD2, CD28, CD30, CD40, CD50 (ICAM3), CD54 (ICAM1), CD80, CD86, B7-H1, CD134 (OX40), CD137 (41BB), CD152 (CTLA-4), CD153 (CD30 ligand), CD154 (CD40 ligand), ICOS, CD19, CD20, CD22, CD37, L6, CD3, CD4, CD25, CD8, CD11b, CD14, CD25, CD56 and CD69.

36. The binding domain-immunoglobulin fusion protein of claim 27 wherein the human IgG is human IgG1.

37. A binding domain-immunoglobulin fusion protein, comprising:

(a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein the binding domain polypeptide is capable of specifically binding to at least one antigen that is present on a cancer cell surface and wherein the hinge region polypeptide comprises a polypeptide selected from the group consisting of a human IgA hinge region polypeptide, a human IgG hinge region polypeptide, and a human IgE hinge region polypeptide;

(b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide, wherein said CH2 constant region polypeptide comprises a polypeptide selected from the group consisting of a human IgA CH2 constant region polypeptide, a human IgG CH2 constant region polypeptide, and a human IgE CH2 constant region polypeptide;

(c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein said CH3 constant region polypeptide comprises a polypeptide selected from the group consisting of a human IgA CH3 constant region polypeptide, a human IgG CH3 constant region polypeptide, and a human IgE CH3 constant region polypeptide; and

(d) a plasma membrane anchor domain polypeptide.

38. The binding domain-immunoglobulin fusion protein of claim 37 wherein the membrane anchor domain polypeptide comprises a transmembrane domain polypeptide.

39. The binding domain-immunoglobulin fusion protein of claim 37 wherein the membrane anchor domain polypeptide comprises a transmembrane domain polypeptide and a cytoplasmic tail polypeptide.

40. The binding domain-immunoglobulin fusion protein of claim 37 wherein the membrane anchor domain polypeptide comprises a glycosyl-phosphatidylinositol-linkage polypeptide sequence.

41. The binding domain-immunoglobulin fusion protein of claim 37 wherein the human IgG is human IgG1.

42. A binding domain-immunoglobulin fusion protein, comprising:

(a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein said hinge region polypeptide comprises a wild-type human IgA hinge region polypeptide;

(b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide, wherein said CH2 constant region polypeptide comprises a human IgA CH2 constant region polypeptide; and

(c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein said CH3 constant region polypeptide comprises a polypeptide selected from the group consisting of (i) a wild-type human IgA CH3 constant region polypeptide and (ii) a mutated human IgA CH3 constant region polypeptide that is incapable of associating with a J chain,

wherein:

(1) the binding domain-immunoglobulin fusion protein is capable of at least one immunological activity selected from the group consisting of antibody dependent cell-mediated cytotoxicity and complement fixation, and

(2) the binding domain polypeptide is capable of specifically binding to an antigen.

43. The binding domain-immunoglobulin fusion protein of claim 42 wherein the mutated human IgA CH3 constant region polypeptide that is incapable of associating with a J chain is selected from the group consisting of (i) a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:_____ and (ii) a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:_____.

44. A binding domain-immunoglobulin fusion protein, comprising:

(a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide;

(b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide, wherein said CH2 constant region polypeptide comprises a llama CH2 constant region polypeptide that is selected from the group consisting of a llama IgG1 CH2 constant region polypeptide, a llama IgG2 CH2 constant region polypeptide and a llama IgG3 CH2 constant region polypeptide; and

(c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein said CH3 constant region polypeptide comprises a llama CH3 constant region polypeptide that is selected from the group consisting of a llama IgG1 CH3 constant region polypeptide, a llama IgG2 CH3 constant region polypeptide and a llama IgG3 CH3 constant region polypeptide

wherein:

(1) the binding domain-immunoglobulin fusion protein is capable of at least one immunological activity selected from the group consisting of antibody dependent cell-mediated cytotoxicity and induction fixation of complement, and

(2) the binding domain polypeptide is capable of specifically binding to an antigen.

45. A binding domain-immunoglobulin fusion protein according to claim 44 wherein the immunoglobulin hinge region polypeptide, the llama CH2 constant region polypeptide and the llama CH3 constant region polypeptide comprise sequences derived from a llama IgG1 polypeptide and wherein the fusion protein does not include a llama IgG1 CH1 domain.

46. A binding domain-immunoglobulin fusion protein according to any one of claims **1**, **23**, **24**, **27**, **37**, **42** or **44** wherein the hinge region polypeptide is mutated to contain a glycosylation site.

47. The fusion protein of claim 46 wherein the glycosylation site is selected from the group consisting of an asparagine-linked glycosylation site, an O-linked glycosylation site, a C-mannosylation site, a glypiation site and a phosphoglycation site.

48. A binding domain-immunoglobulin fusion protein according to any one of claims **1**, **23**, **24**, **27**, **37**, **42** or **44** wherein the binding domain polypeptide comprises two or more binding domain polypeptide sequences wherein each of said binding domain polypeptide sequences is capable of specifically binding to an antigen.

49. A binding domain-immunoglobulin fusion protein, comprising:

(a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein the hinge region polypeptide comprises an alternative hinge region polypeptide sequence;

(b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide; and

(c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide,

wherein:

(1) the binding domain-immunoglobulin fusion protein is capable of at least one immunological activity selected from the group consisting of antibody dependent cell-mediated cytotoxicity and complement fixation, and

(2) the binding domain polypeptide is capable of specifically binding to an antigen.

50. A binding domain-immunoglobulin fusion protein, comprising:

(a) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, wherein the binding domain polypeptide is capable of specifically binding to at least one antigen that is present on a cancer cell surface and wherein the hinge region polypeptide comprises an alternative hinge region polypeptide sequence;

(b) an immunoglobulin heavy chain CH2 constant region polypeptide that is fused to the hinge region polypeptide, wherein said CH2 constant region polypeptide comprises a polypeptide selected from the group consisting of a human IgA CH2 constant region polypep-

tide, a human IgG CH2 constant region polypeptide, and a human IgE CH2 constant region polypeptide;

(c) an immunoglobulin heavy chain CH3 constant region polypeptide that is fused to the CH2 constant region polypeptide, wherein said CH3 constant region polypeptide comprises a polypeptide selected from the group consisting of a human IgA CH3 constant region polypeptide, a human IgG CH3 constant region polypeptide, and a human IgE CH3 constant region polypeptide; and

(d) a plasma membrane anchor domain polypeptide.

51. A binding domain-immunoglobulin fusion protein according to either claim 49 or claim 50 wherein the alternative hinge region polypeptide sequence comprises a polypeptide sequence of at least ten continuous amino acids that are present in a sequence selected from the group consisting of SEQ ID NOS:___-___.

52. An isolated polynucleotide encoding a binding domain-immunoglobulin fusion protein according to any one of claims **1**, **23**, **24**, **27**, **37**, **42**, **44**, **49** and **50**.

53. A recombinant expression construct comprising a polynucleotide according to claim 52 that is operably linked to a promoter.

54. A host cell transformed or transfected with a recombinant expression construct according to claim 53.

55. A method of producing a binding domain-immunoglobulin fusion protein, comprising the steps of:

(a) culturing a host cell according to claim 54 under conditions that permit expression of the binding domain-immunoglobulin fusion protein; and

(b) isolating the binding domain-immunoglobulin fusion protein from the host cell culture.

56. A pharmaceutical composition comprising a binding domain-immunoglobulin fusion protein according to any one of claims **1**, **23**, **24**, **42**, **44**, **49** or **50** in combination with a physiologically acceptable carrier.

57. A pharmaceutical composition comprising an isolated polynucleotide encoding a binding domain-immunoglobulin fusion protein according to any one of claims **1**, **23**, **24**, **27**, **37**, **42**, **44**, **49** and **50** in combination with a physiologically acceptable carrier.

58. A method of treating a subject having or suspected of having a malignant condition or a B-cell disorder, comprising administering to a patient a therapeutically effective amount of a pharmaceutical composition selected from the group consisting of the pharmaceutical composition of claim 56 and the pharmaceutical composition of claim 57.

59. The method of claim 58 wherein the malignant condition or B-cell disorder is selected from the group consisting of a B-cell lymphoma and a disease characterized by autoantibody production.

60. The method of claim 58 wherein the malignant condition or B-cell disorder is selected from the group consisting of rheumatoid arthritis, myasthenia gravis, Grave's disease, type I diabetes mellitus, multiple sclerosis and an autoimmune disease.

61. The method of claim 58 wherein the malignant condition is selected from the group consisting of melanoma, carcinoma and sarcoma.

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VIEWPOINT
IMMUNOLOGY TODAY

Interleukin 15: a proinflammatory role in rheumatoid arthritis synovitis

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In rheumatoid arthritis (RA), locally produced interleukin 15 (IL-15) can recruit and activate synovial T cells, which then amplify and perpetuate inflammation through induction of monocyte-derived tumour necrosis factor α (TNF-α) via a cell-contact-dependent mechanism. Thus, IL-15 could play a pivotal role in disease pathogenesis, acting upstream of TNF-α in orchestrating the induction of a cascade of inflammatory cytokines.

Rheumatoid arthritis (RA) is a chronic inflammatory disease, the etiopathogenesis of which remains unclear. The clinical syndrome is characterized by relapsing–remitting inflammation within the synovial membrane, associated with progressive, erosive destruction of adjacent cartilage and bone. The cellular components present within the synovium comprise T cells, monocyte/macrophages, mast cells, neutrophils and plasma cells, together with an expanded population of activated synovial fibroblasts (synoviocytes). No consensus exists as to which is the principal regulatory cell, representing the optimal therapeutic target, or whether such regulatory contribution is stable with disease progression.

Given the postulated autoimmune basis for RA, most debate surrounds the role of T cells in pathogenesis (reviewed in Ref. 1). Several factors suggest a proinflammatory role for T cells in RA: their critical role in animal arthritis models, the partial efficacy of T-cell-directed therapies in clinical studies and, most persuasively, the disease association with specific human major histocompatibility complex (MHC) HLA-DR alleles. However, alternative explanations for MHC linkage have also been proposed, including 'multistep' molecular mimicry[2]. Furthermore, T-cell-derived cytokines are detected only at low levels in synovial membrane, and bone or cartilage destruction does not necessarily correlate with the presence of synovial inflammation.

The majority of synovial T cells are $CD45RB^{dim}CD45R0^{+}CD27^{-}$, representing a mature, memory T-cell population that expresses a complex combination of early and late activation markers, including multiple adhesion molecules. Although extensive receptor analyses have identified oligoclonality in the synovial T-cell repertoire, as yet there are no consistent data to indicate a single antigen-driven process. Moreover, even the detection of expanded, putative 'autoantigen'-specific T-cell clones would probably account for only a proportion of the total T-cell population observed within the synovial membrane. Several mechanisms might contribute to accumulation of the remaining polyclonal population. $CD45R0^{+}CD27^{-}$ peripheral blood (PB) T cells possess intrinsic transepithelial migratory potential *in vitro*[3], which can account for their preferential recruitment to dermal inflammatory sites *in vivo*[4]. Within synovial membranes, T cells interact with MHC class II and extracellular matrix components through β-integrin expression. Such integrin–ligand interactions may rescue synovial T cells from apoptosis through modulation of *bcl-2* expression[5]. Thus, synovial membrane probably represents a privileged site, within which prolonged T-cell residence may be sustained.

Critical questions remain unanswered. First, are the only proinflammatory synovial T cells those that are specific for 'arthritogen', with remaining T cells representing innocent bystanders? Second, given the absence of significant T-cell cytokine production, particulary interleukin 2 (IL-2) and interferon γ (IFN-γ), by what other means might T cells contribute to the inflammatory process? This article reviews data which demonstrate that synovial T-cell recruitment and activation can occur as a result of local synthesis of IL-15, and that such nonspecific activation can result in perpetuation of inflammation through induction of monocyte-derived tumour necrosis factor α (TNF-α) synthesis via a cell-contact-dependent pathway. The expression, structure and biological activities of IL-15 are reviewed in Box 1.

IL-15 expression in RA

The RA synovial membrane, in which monokine production predominates[15], represents an ideal chronic disease model to elucidate *ex vivo* the regulation and functional significance of IL-15 expression. IL-15 mRNA is detected in RA synovial membrane and its relative expression varies with prior immunosuppressive therapy[16]. IL-15 can be detected by ELISA in RA synovial fluids (SF) in approximately 50% of patients with mean concentration 307 pg ml^{-1} (SE 62, $n = 54$). This activity correlates with SF TNF-α concentrations and remains after removal of rheumatoid factor which, because of its immunoglobulin component, might have interfered with earlier assay systems. IL-15 can also be detected immunohistochemically in the RA synovial membrane, where it is localized to macrophages and fibroblasts in the synovial lining layer and within lymphocytic aggregates[17]. Subsequent studies have also shown IL-15 in synovial biopsies from patients with reactive arthritis, in which synovial T cells also express IL-15 (Ref. 18). It remains unclear whether this represents membrane-bound or synthesized cytokine and *in situ* mRNA studies are awaited. Together, these data clearly demonstrate that IL-15 can be expressed at the mRNA and protein level during synovial inflammation.

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Box 1. Interleukin 15

Expression and structure

IL-15 is a novel 14–15 kDa cytokine expressed at least at the mRNA level in numerous normal human tissues, including skeletal muscle and kidney, as well as in cell types such as activated monocytes and fibroblasts[6–8].

IL-15 consists of the IL-2 receptor (IL-2R) β-chain and γ-chain, together with a unique α-chain (IL-15Rα), which is alternatively spliced to yield three active forms, each capable of high-affinity binding to IL-15. Whereas IL-2Rα is primarily expressed on activated T cells, IL-15Rα mRNA has been identified in numerous human tissues and cell lines, including activated T cells. Since IL-15Rα alone does not transduce signal in transfected cells, its functional significance in the absence of βγ-chain expression is unclear, but may include scavenging of IL-15 at inflammatory sites. A novel 60–65 kDa receptor for IL-15 has been identified on mast cells, designated IL-15RX, which requires neither IL-2Rβ nor IL-2Rγ for signalling[8]. This receptor recruits distinct signalling pathways. Whereas the IL-15αβγ complex signals through JAK1/3 and STAT3/5, IL-15RX utilizes JAK2 and STAT5.

Bioactivity

Multiple biological effects of IL-15 have been described (Fig. 1). It induces proliferation of mitogen-activated CD4⁺ and CD8⁺ T cells, T-cell clones and γδ T cells, with release of soluble IL-2Rs, and enhanced cytotoxicity both in CD8⁺ T cells and lymphokine-activated killer cells[6–8]. CD69 expression is upregulated on CD45R0⁺ but not CD45RA⁺ T-cell subsets, consistent with the distribution of IL-2Rβ expression. Whether IL-15 prejudices T helper 1 (Th1) or Th2 differentiation is unclear. IL-15 primes naive CD4⁺ T cells from TCR-transgenic mice for subsequent IFN-γ expression, but not IL-4 production; furthermore, antigen-specific responses in T cells from human immunodeficiency virus (HIV)⁺ patients in the presence of high-dose IL-15 exhibit increased IFN-γ production, particularly if IL-12 is relatively deficient[9,10]. However, IL-15 induces IL-5 production from allergen-specific human T-cell clones, implying a positive role in Th2-mediated allergic responses[11]. Thus, through its function as a T-cell growth factor, IL-15 probably sustains either Th1 or Th2 polarization. IL-15 is also a potent T-cell chemokine[12] and induces adhesion molecule redistribution to uropods in the presence of β-integrin ligands[13]. Thus, IL-15 can recruit T cells and, thereafter, modify homo- or heterotypic cell–cell interactions within inflammatory sites.

Further bioactivities have potential relevance to inflammatory arthritis (reviewed in Ref. 8). IL-15 supports B-cell proliferation and immunoglobulin synthesis *in vitro*, in combination either with CD40 ligand (CD40L), or immobilized anti-IgM. Recently, neutrophil activation, cytoskeletal rearrangement and protection from apoptosis by IL-15 has been reported, as has induction of mast cell proliferation[14]. IL-15 induces natural killer (NK)-cell activation, measured either by direct cytotoxicity, antibody-dependent cellular cytotoxicity or production of cytokines. Moreover, several reports demonstrate a role for IL-15 in thymic development of T-cell and, particularly, NK-cell lineages. The widespread tissue distribution of mRNA for IL-15 and IL-15Rα indicates that diverse functions may exist beyond the immune system, including anabolic nonproliferative effects in skeletal muscle *in vitro*. IL-15 apparently represents a mechanism whereby host tissues can contribute to the early phase of immune responses, providing enhancement of polymorphonuclear and NK-cell responses, and subsequently T-cell responses, prior to optimal IL-2 production. The corollary to such pleiotropic activity may be a propensity to chronic, rather than self-limiting, inflammation should IL-15 synthesis be aberrantly regulated.

Investigation of the functional consequences of IL-15 expression indicates a central role in synovial T-cell recruitment and activation. RA SF contains chemotactic activity for PB lymphocytes, demonstrated in polarization and matrix migration assays; this activity is attributable to the presence of IL-15, together with macrophage inflammatory protein 1α (MIP-1α), monocyte chemotactic protein 1 (MCP-1) and IL-8 (Ref. 19). Those PB T cells that respond in chemotactic assays to RA SF, or to recombinant (r)IL-15, are of the CD45R0⁺ subset. *In vivo* studies in mice primed with either *Corynebacterium parvum*, or type II bovine collagen, confirm that such chemotactic activity is proinflammatory, since those animals that receive rIL-15 via footpad injections develop local tissue swelling, T-cell accumulation and local lymphadenopathy (Ref. 17; B.P. Leung and I.B. McInnes, unpublished). PB lymphocytes from RA patients rapidly upregulate CD69 expression and exhibit enhanced responsiveness to IL-15 in proliferation assays in comparison to normal age/gender-matched controls. SF T cells contain IL-15Rα mRNA (I.B. McInnes and F.Y. Liew, unpublished) and retain the ability to proliferate vigorously to rIL-15, in contrast to their reduced responses to mitogens such as phytohemagglutinin (PHA). Together, these data indicate that IL-15 can both recruit T cells to, and activate them within, the synovial compartment.

IL-15 and TNF-α

Data from animal arthritis models, *in vitro* synovial cultures and recent clinical trials in RA patients indicate that TNF-α occupies a pivotal position in the regulation of synovial inflammation[15]. However, less is known about the factors that in turn upregulate TNF-α synthesis, particularly in the relative absence of IFN-γ.

Although IL-15 induces TNF-α production by SF T cells *in vitro*[20], it appears not to have a direct effect on macrophages, which represent the predominant source of TNF-α *in vivo*. Immunohistochemical studies clearly demonstrate juxtaposition of T cells and macrophages in RA synovial membrane, with concomitant, reciprocal adhesion molecule expression, suggestive of regulatory 'cross-talk'. The question arises whether IL-15 might induce macrophage activation through cognate interactions with activated T cells. Following stimulation with nonphysiological mitogens, paraformaldehyde-fixed T cells and T-cell clones induce proinflammatory cytokine production by macrophages and fibroblasts through cell contact[21]. Using a similar assay system, it was shown that freshly isolated synovial T cells induce TNF-α

synthesis by blood- or synovial-derived macrophages *ex vivo* through cell-membrane contact, with no requirement for secretory factors[20]. This activity is maintained *in vitro* by addition of rIL-15 but not by rIL-2. Moreover, rIL-15 confers similar properties upon CD45R0⁺ PB T cells, such that rIL-15-activated PB T cells from RA patients induce TNF-α synthesis in synovial macrophage/synoviocyte co-cultures. Neutralization studies implicate at least CD69, leukocyte function-associated molecule 1 (LFA-1) and intercellular adhesion molecule (ICAM-1) in this pathway. Thus, these data provide evidence that IL-15 is an important enhancer of TNF-α production in RA synovial membranes.

A critical role for IL-15 in synovial inflammation?

In the absence of a clearly defined antigen, antigen-independent proinflammatory processes within the RA synovial membrane offer the best targets for therapeutic intervention. We have proposed that IL-15 occupies a central position in the perpetuation of synovial inflammation in RA (Fig. 2)[20]. Thus, IL-15 can recruit and expand CD45R0⁺ memory T-cell subsets in the synovial membrane, in which, under the continuing influence of IL-15, newly recruited T cells can produce TNF-α directly or via cell contact with macrophages. This may generate a positive-feedback loop, whereby IL-15 synthesis by activated synovial macrophages or fibroblasts induces continued T-cell recruitment, with consequent maintenance of macrophage activation and TNF-α production through further cell contact. Additional effects of IL-15 on local B-cell immunoglobulin secretion, particularly rheumatoid factor synthesis, and on neutrophil activation are also predicted. This hypothesis places IL-15 upstream from TNF-α in the cytokine hierarchy within the synovial membrane and provides a mechanism for the amplification of inflammation by T cells. The latter need not be directed primarily by local recognition of antigen, and may proceed in the relative absence of T-cell-derived cytokines, particularly IFN-γ.

Cytokine-mediated nonspecific activation of T cells has been observed. Polyclonal T-cell activation follows injection of type I IFN *in vivo* in mice[22] and resting human CD4⁺ T cells can be activated to produce cytokines and provide B-cell help *in vitro* by a combination of IL-2, IL-1β and TNF-α (Ref. 23). The detection of IL-15 in synovial membrane allows the presence of a similar proinflammatory mechanism in RA to be reconciled with the relative absence of IL-2. IL-15 is unlikely to act in isolation within the synovial milieu (Fig. 2). TNF-α and IL-6 together enhance the capacity of IL-15 to induce cell-contact-mediated macrophage activation[24]. Moreover, the continued presence of soluble cytokines may further modify the effects of cognate interactions with target cells. For example, granulocyte–macrophage colony-stimulating factor (GM-CSF) enhances cytokine-activated, T-cell-induced TNF-α synthesis by blood-derived monocytes[24], whereas IL-10 is inhibitory. By this means, the soluble or cell-bound cytokine products of cognate interactions may feedback within the system to 'fine-tune' macrophage activation. Thus, a complex series of interactions probably exists in the synovial membrane, the balance of which will ultimately determine the 'pro-' versus 'anti-' inflammatory effect of the T cell–macrophage contact.

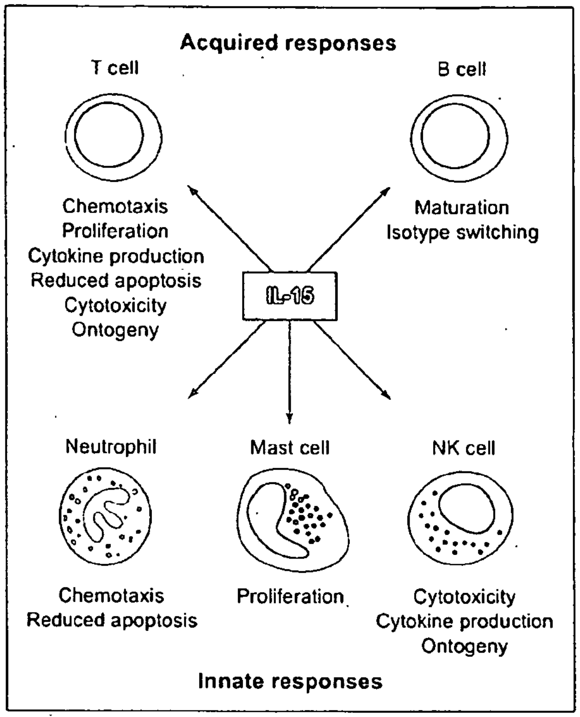

Fig. 1. *Biological effects of IL-15 with potential relevance to rheumatoid arthritis synovitis. Abbreviations: IL-15, interleukin 15; NK, natural killer.*

Activation without antigen?

Antigen-independent T-cell responses apparently contradict the two-signal requirement for T-cell activation [T-cell receptor (TCR)–peptide–MHC and costimulation]. However, interactions of CD45R0⁺ T cells with 'bystander' cytokines, adhesion molecules and, perhaps, crossreactive antigens, as might occur during occasional infectious episodes, have been proposed as pathways whereby the T-cell memory compartment is maintained in the absence of initiating antigen[22,25]. Thus, although some IL-15-responsive lymphocytes may be recirculating, having recently recognized endogenous or exogenous antigen *in vivo*, others may receive signals within the synovial environment that facilitate continued responsiveness to IL-15. Upregulated HLA class II expression in synovial membrane might provide such a stimulus. It is intriguing that transgenic mice with TCR specificity for autologous MHC develop a progressive, erosive arthropathy, which closely resembles RA and is T-cell dependent[26].

Inflammatory processes that are not driven by antigen may complement an autoantigen-specific response. Synovial T cells are placed at the centre of a web of cognate interactions, which extends beyond monocytes to include synovial fibroblasts, B cells and endothelial cells. For example, T-cell-contact-mediated activation of fibroblasts leads to cytokine and metalloproteinase secretion[21]. Thus, diverse cell types within synovial membrane may exhibit coordinate proinflammatory activities through cell contact. Elucidation of such mechanisms should yield clinical strategies for treating RA. T-cell-directed therapies that not only inhibit T-cell activation but also deplete T cells from the synovial compartment, or at least interfere with their membrane interactions, will probably be most

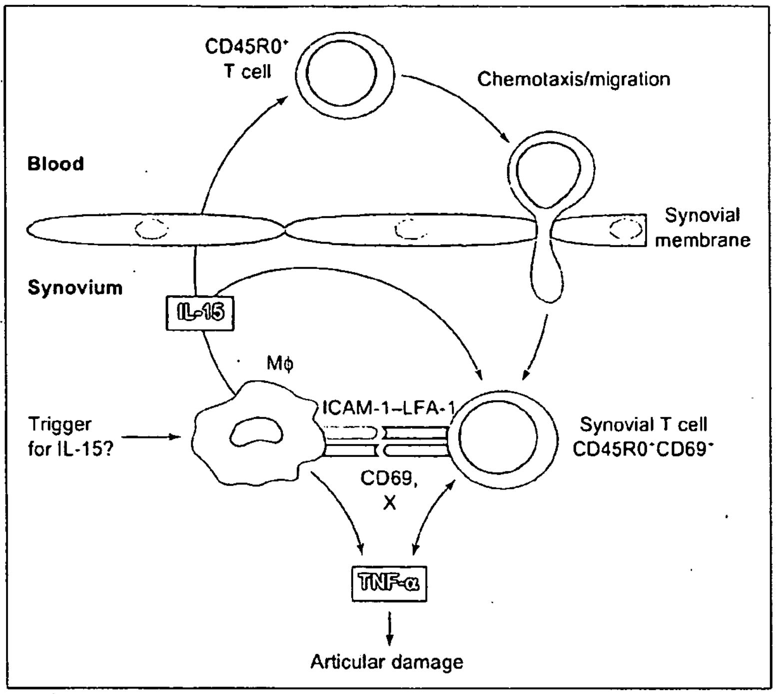

Fig. 2. *IL-15-induced amplification of rheumatoid arthritis synovitis. Synovial macrophage- or fibroblast- (not shown) derived IL-15 mediates recruitment and activation of T cells and confers upon them the capacity for macrophage activation/TNF-α production through cell contact, in the absence of significant IFN-γ synthesis. Such TNF-α synthesis is crucial for downstream activation of numerous proinflammatory pathways that lead to joint destruction in RA. For clarity, the roles of other cytokines (e.g. IL-1β, IL-6 and IL-10) and mediators (e.g. metalloproteinases, prostaglandins) are not shown; X indicates unknown adhesion molecule(s) (see Ref. 19). Abbreviations: ICAM-1, intercellular adhesion molecule 1; IFN-γ, interferon γ; IL, interleukin; LFA-1, leukocyte function-associated molecule 1; Mϕ, macrophage; TNF-α, tumour necrosis factor α.*

efficacious. It is of interest that clinical improvement following anti-CD4 therapy in RA correlates with synovial T-cell coating with anti-CD4 (Ref. 27).

Regulation of IL-15 expression

Intriguing questions arise from these observations. Of most importance are those surrounding the factors that initiate and perpetuate IL-15 synthesis in RA. The widespread detection of IL-15 mRNA has not consistently correlated with IL-15 protein expression. Factors demonstrated thus far to induce IL-15 secretion by human cells are diverse and include human herpesvirus 6, *Mycobacterium leprae*, staphylococcus, lipopolysaccharide and ultraviolet irradiation[8,28]. Whereas many cytokines are regulated by modification of transcription and message stabilization, IL-15 appears subject to significant post-transcriptional regulation. The IL-15 mRNA 5'-untranslated region (UTR) contains ten AUG triplets that significantly reduce the efficiency of translation. Fusion of the IL-15 mRNA with a human T-cell leukaemia virus 1 (HTLV-1) R region in the HuT-102 cell line deleted this AUG-rich 5'UTR sequence, leading to high levels of constitutive IL-15 secretion[5]. Replacement of the IL-15 signal peptide with that of IL-2 or CD33 induces significantly higher levels of IL-15 production in transfection systems, indicating that this region is also normally involved in downregulating IL-15 protein release[8,29]. These multiple levels of regulation presumably provide an available pool of mRNA, a suggestion that is compatible with the perceived early functional role of IL-15, but under normal circumstances prevent undesirable IL-15 expression in tissues. The RA synovial membrane represents an attractive site at which to study the mechanisms whereby continued IL-15 secretion is aberrantly facilitated *in vivo*.

Therapeutic implications

Bystander cytokine-mediated T-cell contact might represent a general mechanism whereby polyclonal T-cell recruitment can contribute to ongoing inflammation. Bioactivities described for neutrophils and natural killer cells indicate a key role for IL-15 early during immune responses, with transient expression thereafter to limit inflammatory effects. Failure to downregulate such IL-15 expression appropriately, or continued upregulation by unknown factors, could therefore lead to chronicity rather than resolution of inflammatory foci. 'Pathological' IL-15 expression has now been described in several chronic diseases, including pulmonary sarcoid[30], asthma (B. Leung and I.B. McInnes, unpublished), leprosy[31] and ulcerative colitis[32] and, in the latter, this expression was correlated with disease activity. The efficacy of TNF-α blockade in RA has elegantly demonstrated the therapeutic utility, albeit transient, of cytokine-directed therapy in the treatment of chronic inflammatory disease[15]. The identification of IL-15-mediated T-cell and monocyte activation in the synovial membrane, apparently operating upstream from the effects of TNF-α, provides a novel target for such biological therapeutic approaches. This might be either through direct neutralization of IL-15 or by targeting IL-15 receptors, particularly IL-15Rα. Studies in animal models of arthritis are now required to address these exciting possibilities.

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Calling all Glasgow University immunology graduates

It is now 21 years since the first students graduated from what was then Britain's only BSc Honours degree in immunology. To mark this coming-of-age, a reunion meeting will be held in Glasgow on **Saturday 2 May 1998**. There will be talks by staff and former students, followed by a dinner.

For further information, please contact Dr Jeremy Brock (Tel: 0141 211 2153; Fax: 0141 337 3217; E-mail: jhb1h@clinmed.gla.ac.uk) or Dr Allan Mowat (Tel: 0141 211 2498; E-mail: amm1u@clinmed.gla.ac.uk) at the Dept of Immunology, Western Infirmary, Glasgow, UK G11 6NT.

Index!

The 1997 index is enclosed with this issue of *Immunology Today*. We hope that you find it a useful resource.

Author's correction

Ghetie, V. and Ward, E.S. (1997) FcRn: the MHC class I-related receptor that is more than an IgG transporter *Immunology Today* 18 (12), 592–598

In Fig. 6, the lysosomal compartments should have contained short red lines, depicting degraded IgG, rather than short green lines. Furthermore, the phrase 'Unbound FcRn degraded' should have read 'Unbound IgG degraded'.

The authors and Editorial staff apologize to the readers for any confusion this might have caused.

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Interleukin-15 mediates T cell-dependent regulation of tumor necrosis factor-α production in rheumatoid arthritis

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Tumor necrosis factor-α occupies a central role in rheumatoid arthritis (RA) pathogenesis. We now report that interleukin-15 (IL-15) can induce TNF-α production in RA through activation of synovial T cells. Peripheral blood (PB) T cells activated by IL-15 induced significant TNF-α production by macrophages via a cell-contact-dependent mechanism. Freshly isolated RA synovial T cells possessed similar capability, and *in vitro*, IL-15 was necessary to maintain this activity. IL-15 also induced direct TNF-α production by synovial T cells. In contrast, IL-2 induced significantly lower TNF-α production in either cell-contact-dependent or direct culture, and IL-8 and MIP-1α were ineffective. Antibodies against CD69, LFA-1 or ICAM-1 significantly inhibited the ability of T cells to activate macrophages by cell contact.

Neither the initiating events, nor the perpetuating factors in the pathogenesis of rheumatoid arthritis (RA) are well understood. The disease is characterized by chronic infiltration of the synovial membrane by T lymphocytes, plasma cells and macrophages. Together with activated fibroblast-like synoviocytes, this constitutes pannus, an invasive tissue capable of eroding into adjacent cartilage and bone, subsequently leading to joint failure[1]. Although these appearances resemble an ongoing autoimmune response, the antigen responsible remains elusive and additional non-antigen-dependent processes have been proposed to explain these features[2].

Within the RA synovial membrane, high levels of proinflammatory cytokines are detectable, particularly tumor necrosis factor-α (TNF-α), interleukins IL-1β and IL-6, granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-8 (reviewed in ref. 3), leading to the suggestion that RA is predominantly a "macrophage-led" process[4]. T cell-derived cytokines such as interferon-γ (IFN-γ), IL-2 and IL-4 may be detected at the mRNA and protein level, but are only found with difficulty and at low cellular frequency[5-7]. These observations have raised questions as to the functional contribution of T lymphocytes to the chronic phase of RA (ref. 8). Nevertheless, several animal arthritis models are T cell-dependent[9], and clinical benefit in RA has been ascribed to anti-T cell therapies such as cyclosporin A (ref. 10). The most persuasive evidence for T-cell involvement in chronic synovitis is provided by the association of disease severity with human leukocyte antigen HLA-DR subtypes[11]. The precise role of T cells in RA, however, remains unclear.

We recently identified interleukin 15 (IL-15) within the synovial membrane of RA patients[12]. IL-15 is a pleiotropic cytokine, derived from several cell types including macrophages and fibroblasts[13,14], which mediates its activity through a heterotrimeric receptor consisting of a unique IL-15Rα chain, in combination with the β and γ chains of the IL-2 receptor[15,16]. IL-15 can induce T-cell proliferation[13], B-cell maturation and isotype switching[17], NK-cell cytotoxicity and cytokine generation[18] and may protect T cells from apoptosis[19]. IL-15 induces inflammatory cell recruitment *in vivo* following injection into the foot pads of mice primed with *Corynebacterium parvum*[12] and in chemotactic assays *in vitro*, T-cell polarization and invasion into collagen gels[20]. Responding migratory T cells are mainly of the CD45RO+ "memory" phenotype[21]. Moreover, RA synovial fluid contains potent chemotactic activity attributable, at least in part, to the presence of IL-15 (ref. 12). We have therefore suggested that IL-15 plays a critical role in T-cell recruitment and activation in RA in the relative absence of IL-2.

The relationship of IL-15 to other cytokines in the RA synovial membrane is currently unclear. Studies in animal models have generated a hypothetical hierarchy of cytokine activities in RA (ref. 3). TNF-α appears pivotal in regulating synthesis of other proinflammatory cytokines, particularly IL-1β, which in turn is important in enhancing chondrocyte bioactivity and the production of matrix metalloproteinases (MMPs)[22]. Furthermore, in RA patients, monoclonal antibodies against TNF-α reduce clinical inflammation and laboratory parameters of disease activity[23,24]. However, it is unclear which factors upregulate TNF-α production in the synovium, particularly in the relative absence of IFN-γ. Non-cytokine-dependent mechanisms may therefore be important. Following mitogen stimulation *in vitro*, T lymphocytes can induce macrophage production of cytokines and MMPs by cell contact[25]. However, it has not previously been possible to extend these observations in the context of RA, because no physiologically relevant T-cell activation factor had been described prior to the identification of IL-15.

We therefore addressed the possibility that IL-15 may induce TNF-α production in RA. We now report that IL-15-activated blood-derived or synovial T cells induced TNF-α production by a macrophage cell line and by RA blood- or synovium-derived monocytes/macrophages. Cell contact was obligatory, mediated in part through expression of leukocyte function-associated antigen (LFA-1), intracellular cell-adhesion molecule (ICAM-1) and T-cell differentiation antigen CD69. These data provide the first evidence of a role for IL-15 in regulation of TNF-α production within the RA synovial membrane. They indicate that polyclonal

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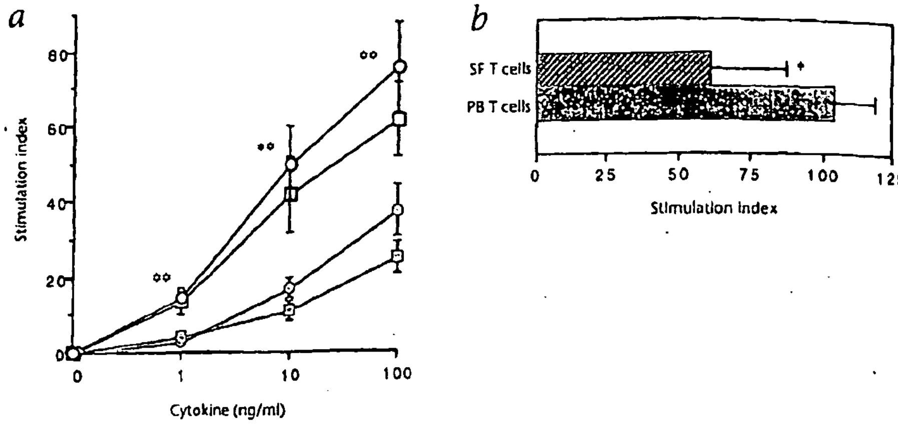


Fig. 1 Synovial lymphocytes proliferate to rIL-15 (circles) and rIL-2 (squares). *a*, T cells from peripheral blood (PB, filled symbols) or synovial fluid (SF, empty symbols) from 15 RA patients were stimulated with doses indicated of either cytokine for 72 h. SF responses exceeded those of PB for both cytokines (** $P < 0.01$). *b*, In contrast, responses to PHA were diminished for SF-derived, compared with PB-derived, T cells (* $P < 0.05$). Data are means ± s.e.m.

synovial T cells, under the direction of IL-15, can exert proinflammatory effects through a cell-contact-dependent mechanism, in the absence of local antigen recognition or cytokine secretion.

Activation of synovial T cells by IL-15

We first compared the proliferative response to recombinant IL-15 (rIL-15) and rIL-2 of T cells derived from matched peripheral blood (PB) and synovial fluid (SF) samples from 15 RA patients. T lymphocyte-enriched populations (>92% $CD3^+$) from both sources proliferated vigorously to rIL-15 and rIL-2 in a similar dose-dependent manner (Fig. 1*a*). However, the response of T cells from the SF was significantly higher than that of T cells from PB. By comparison, the proliferative response of T cells from SF to the T-cell mitogen phytohemagglutinin (PHA) was markedly lower than that of T cells from PB (Fig. 1*b*). These results therefore clearly establish that RA synovial T cells exhibit upregulated responses to IL-15.

We next investigated the consequence of activation of synovial T cells by IL-15. Since TNF-α plays a pivotal role in the pathogenesis of RA (ref. 3), we tested the possibility that IL-15 may upregulate TNF-α synthesis through synovial T cells. T cells from 13 SF samples produced significant amounts of TNF-α in response to IL-15 ($P < 0.005$). In contrast, IL-2 induced only low and variable levels of TNF-α synthesis (Fig. 2*a*). The induction of TNF-α production from synovial T cells was dose dependent (Fig. 2*b*), thus the reduced response to IL-2 was unlikely to be due to an altered dose response. Neither cytokine, however, induced significant TNF-α synthesis by T cells derived from PB (Fig. 2*a*). In comparison, PHA induced TNF-α synthesis by lymphocytes from both SF and PB, but that from PB exceeded that from SF [386 ± 110 pg/ml vs. 184 ± 39 pg/ml, respectively (mean ± s.e.m), $P < 0.03$]. These results therefore demonstrate that synovial T cells can contribute directly to TNF-α production in the RA synovial membrane in response to IL-15. Moreover, they show that the functional effects of IL-15 and IL-2 on this pathological T-cell population may be different.

IL-15-dependent control of TNF-α production by macrophages

As they are the major source of TNF-α in RA synovitis[3], we investigated the effect of IL-15 on TNF-α production by macrophages. Addition of rIL-15 to cultures of macrophage cell lines (U937 and THP-1) or blood-derived monocytes failed to induce TNF-α production (data not shown). These data implied that in RA, IL-15 might mediate its effects on macrophages primarily via T cells. We therefore investigated whether IL-15-activated T cells could induce TNF-α synthesis by macrophages/monocytes. TNF-α production was observed only in cultures in which IL-15-stimulated T cells and macrophages were in direct contact (Fig. 3). As expected, T cells stimulated with PHA/PMA (PHA/phorbol 12-myristate 13-acetate) induced TNF-α production in the presence or absence of cell contact (Fig. 3). These data indicated that a

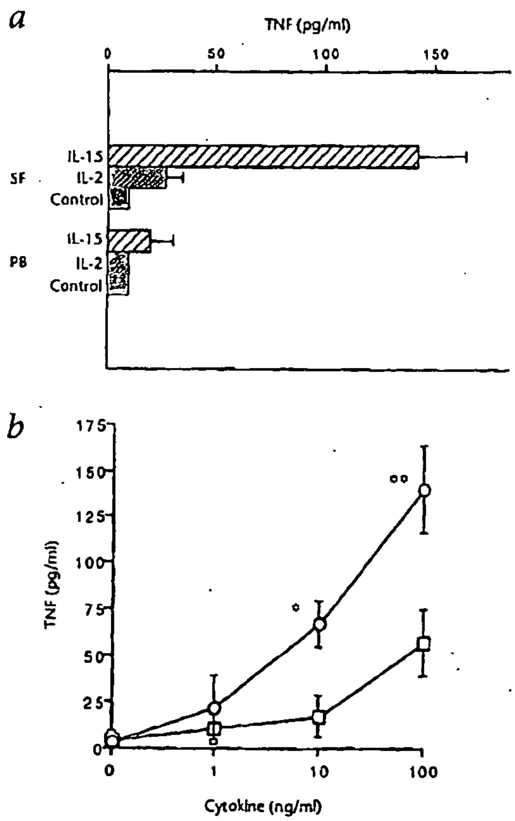


Fig. 2 Tumor necrosis factor-α production by T cell-enriched populations from matched SF and PB. *a*, 13 RA samples were stimulated with rIL-15 (100 ng/ml) or rIL-2 (100 ng/ml) for 72 h. *b*, Dose response to either cytokine of 7 RA SF samples in which IL-2 responses were measurable. rIL-15 (○) induced enhanced levels of TNF-α synthesis from SF lymphocytes compared with rIL-2 (□) (** $P < 0.003$, * $P < 0.03$). Data are means ± s.e.m.

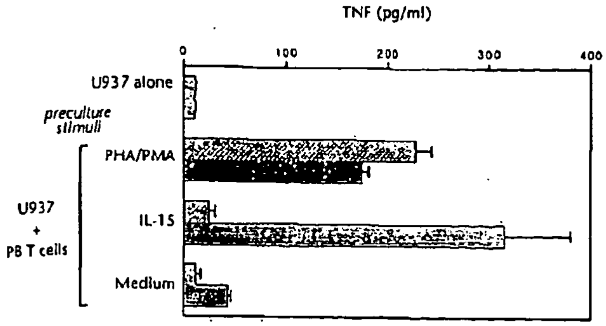
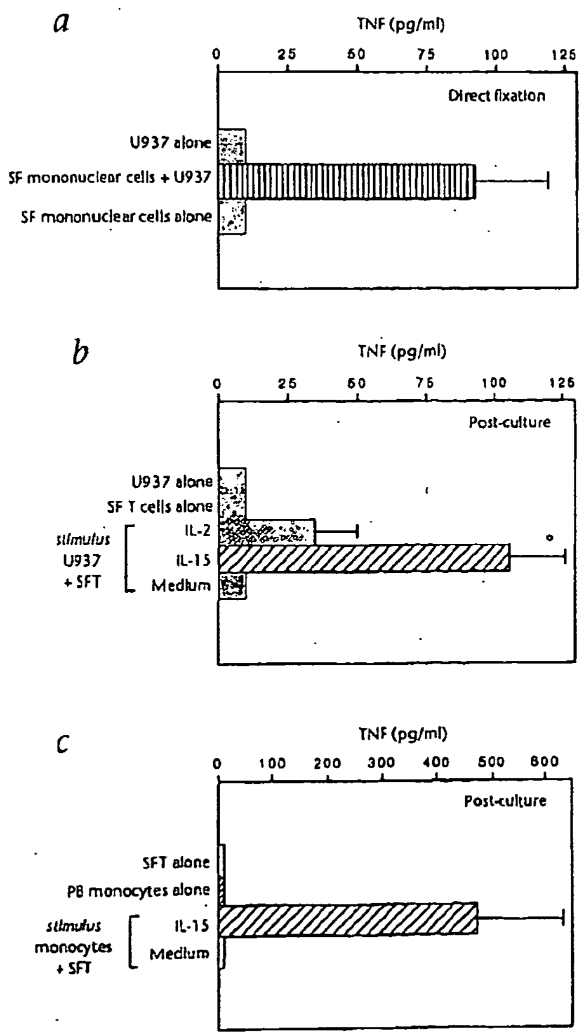


Fig. 3 T-cell/macrophage cell contact is required for IL-15-induced TNF-α production. PHA- (1 μg/ml)/ PMA- (1 nM) or IL-15- (100 ng/ml) activated PB T cells were cultured in contact with (solid bars), or separated by semipermeable membrane from (hatched bars), U937 cells. IL-15-activated T cells required cell contact to induce TNF-α production by U937 cells. Data are means ± s.e.m. of triplicate cultures, representative of three similar experiments.

cell-contact event between IL-15-activated T cells and macrophages was capable of inducing TNF-α production.

To clarify whether T-cell cytokine production was required in addition to cell contact, and to confirm that macrophages were the primary source of TNF-α in these mixed cultures, T cells were fixed in 1% paraformaldehyde (PFA) before coculture with macrophages. PFA fixation is known to prevent T-cell cytokine production but to preserve cell-membrane integrity, providing a simple experimental system for investigation of cell-contact-mediated effects[31]. PFA-fixed T cells from PB of normal individuals (n = 10), previously stimulated with IL-15, consistently induced production of significant concentrations of TNF-α by U937 cells (186.2 ± 17.3 pg/ml). In contrast, similar prior activation of T cells with IL-2 was effective in inducing TNF-α production only in some donors and did so at lower levels (32.8 ± 12.1 pg/ml, $P < 0.005$ compared with IL-15-activated PB T cells). This is unlikely to be due to a dose–response effect, as the concentration of IL-2 used (100 ng/ml) was supra-optimal, and because IL-2 and IL-15 had identical induction profiles for T-cell proliferation (Fig. 1*a*) and polarization[20]. Moreover, lower concentrations of IL-2 were not effective at inducing macrophage activation. To investigate whether other T-cell chemotactic factors might possess activity similar to that of IL-15, we stimulated PB T cells with IL-8 or macrophage inflammatory protein (MIP-1α) at doses known to induce polarization and migration, but no TNF-α production was detected (data not shown). IL-15-activated, PFA-fixed T cells alone were unable to produce TNF-α, even after addition of further PHA (1 μg/ml), indicating that macrophages were the source of TNF-α in the cocultures. Identical results were obtained when highly purified syngeneic peripheral blood-derived monocytes were used instead of U937 cells (131.8 ± 24.7 pg/ml), demonstrating that these results are not likely to be due to an allogeneic effect between T cells and macrophages, nor could it be a unique feature of an immortalized macrophage cell line. Finally, separation of IL-15-activated, PFA-fixed T cells from U937 cells in double-chamber wells prevented induction of TNF-α production (data not shown), confirming that a soluble factor was not involved after fixation.



Induction of TNF-α production by cells of synovial origin

In order to determine whether a similar mechanism might operate *in vivo* in RA, freshly isolated T cell-enriched synovial fluid mononuclear cells from RA patients (n = 8) were fixed with PFA and then added to U937 cells. Significant TNF-α production was observed after 48 hours in all culture supernatants (Fig. 4*a*), demonstrating that synovial T cells may have been sufficiently activated by IL-15 *in vivo* to induce TNF-α production by macrophages. We next investigated whether IL-15 was required to maintain this ability of synovial T cells to induce macrophage TNF-α synthesis. TNF-α was produced only when synovial T cells were maintained in IL-15 or, to a significantly lesser extent, in IL-2 ($P < 0.05$, Fig. 4*b*). Similar results were obtained using syngeneic peripheral blood monocytes (instead of U937 cells) which were obtained from each RA patient at the time of joint aspiration (Fig. 4*c*). Finally, to confirm that synovial macrophage

Fig. 4 Interleukin-15 is required to sustain synovial T cell-mediated cell-contact induction of TNF-α synthesis. PFA-fixed SF T cells from RA patients induced TNF-α synthesis by U937 cells, whether (*a*) fixed immediately (n = 8), or (*b*) after culture for 16 h (n = 10) in the presence or absence of rIL-15 (100 ng/ml). Culture with rIL-2 (100 ng/ml) induced significantly less TNF-α synthesis (*$P < 0.01$ compared with IL-15). *c*, PFA-fixed SF T cells from RA patients induced TNF-α synthesis by RA blood monocytes from the same donor patients (n = 3). Data are means ± s.e.m.

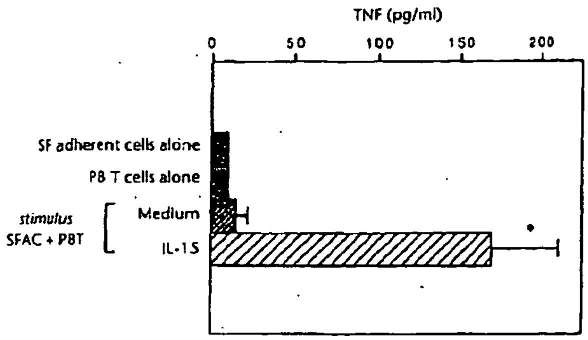


Fig. 5 Interleukin-15-activated T cells induce TNF-α synthesis by synovial macrophage/synoviocyte cocultures (*$P < 0.01$). PB T cells from RA patients ($n = 4$) were stimulated with medium alone or IL-15 (100 ng/ml) for 72 h, then PFA-fixed before addition to synovial macrophage/synoviocyte cocultures. Data are means ± s.e.m.

TNF-α synthesis could be enhanced by this pathway, T cells from PB of four RA patients were added to syngeneic synovial macrophage/synoviocyte cocultures. TNF-α synthesis was observed only with prior activation of PB T cells by IL-15 (Fig. 5). These data clearly demonstrate that synovial T cell-mediated upregulation of TNF-α production by macrophages can occur in RA, and they indicate that IL-15 within the synovial membrane enhances this activity.

Markers required for cell-contact-dependent TNF-α synthesis

Initial experiments characterized alterations in cell-surface phenotype following activation with rIL-15 compared with rIL-2. Both IL-15 and IL-2 upregulated CD69 expression on CD3+ lymphocytes, which was detectable in whole-blood cultures up to 72 hours ($P < 0.005$, Fig. 6*a*). Because circulating T cells recruited to synovial membrane are predominantly of "memory" phenotype[24], we next examined the effect of IL-15 on CD69 expression in the CD45RO+ subset. CD69 expression was elevated within 24 hours and enhanced sevenfold in CD45RO+ PB T cells by 72 hours after addition of rIL-15 *in vitro* ($P < 0.02$, Fig. 6*b*). In contrast, CD45RA+ cells demonstrated twofold enhancement only of CD69 levels, which was accounted for by elevated CD69 expression on CD45RA+, CD16+, CD56+ NK cells. Neither LFA-1 nor ICAM-1 levels on PB T lymphocytes were altered up to 72 hours after addition of IL-15 (data not shown).

We therefore investigated the contribution of CD69, in addition to the adhesion molecules LFA-1 and ICAM-1, to the production of TNF-α by monocytes after contact with IL-15-activated T cells. TNF-α production by PB T-cell/U937 cocultures ($n = 4$) was significantly reduced by neutralization of LFA-1 ($P < 0.02$) or ICAM-1 ($P < 0.03$) and almost completely abrogated by addition of anti-CD69 antibody ($P < 0.001$, Fig. 7*a*). Parallel experiments demonstrated similar involvement of CD69 ($P < 0.001$), LFA-1 and ICAM-1 (both $P < 0.01$) on the production of TNF-α by blood-derived monocytes induced by IL-15-activated T cells (Fig. 7*b*). Addition of human IgG to minimize Fc receptor binding on U937 cells and blood monocytes by T cell-surface bound antibody did not reverse the inhibition of TNF-α production by macrophages.

Discussion

Interleukin-15 induces TNF-α production in RA. IL-15-stimulated proliferation and direct TNF-α production in T cells derived from synovial fluid was enhanced compared with those from blood, thereby establishing that T-cell responsiveness to IL-15 was upregulated *in vivo*. However, T cells represent only a minor source of TNF-α compared with macrophages in RA synovium[17]. IL-15-activated PB T cells induced significant TNF-α production from either unprimed U937 cells, syngeneic blood-derived monocytes or RA synovial macrophage/synoviocyte cultures, by a cell-contact-dependent mechanism. Freshly isolated SF T cells behaved like IL-15-activated PB T cells, indicating that this pathway could operate *in vivo* in RA. IL-15 was required to maintain this activity *in vitro*, because synovial T cells cultured in the absence of IL-15 for 16 hours lost this ability. This is unlikely to be simply the result of apoptosis due to the absence of IL-15, because IL-2, which can rescue T cells from apoptosis *in vitro*[25], was incapable of maintaining synovial T cell-contact activity. Because recently recruited CD14+ monocyte/macrophages constitute a major source of TNF-α in the synovial membrane[27], these data together provide compelling evidence for T cell-contact-mediated upregulation of TNF-α synthesis by macrophages, driven by IL-15 produced in the synovial membrane.

Polyclonal T-cell activation by IL-15. Proinflammatory cytokine and metalloproteinase production following cell-contact-mediated activation of macrophages and fibroblasts by T cells stimulated with nonphysiological mitogens (PHA, PMA, OKT3) has been reported[11]. Here we have used a cytokine widely distributed in the RA synovial membrane[17]. Synovial T cells are predominantly CD45RO+, RB^{dim}, CD27-, implying advanced differentiation[28]. They often simultaneously express early (CD69), mid (HLA-DR) and late (VLA1) markers of activation[28-30], perhaps initiated by interactions with endothelium during extravasation

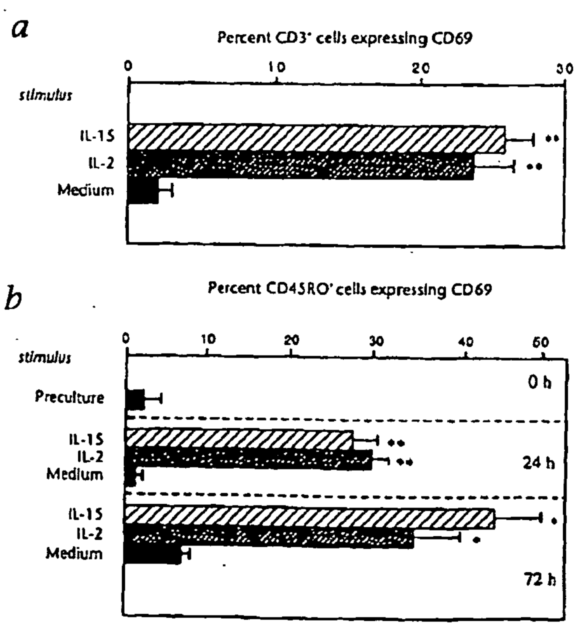


Fig. 6 Interleukin-15 upregulates expression of CD69 on PB T cells. *a*, CD69 expression on CD3+ PB T cells stimulated with IL-15 (100 ng/ml) or IL-2 (100 ng/ml) *in vitro* for 72 h ($n = 6$, **$P < 0.005$ compared with medium alone). *b*, CD69 expression was enhanced in CD45RO+ T cells up to 72 h (*$P < 0.02$, **$P < 0.002$ compared to medium alone) by addition of IL-15 (100 ng/ml) or IL-2 (100 ng/ml) *in vitro*. No significant difference between IL-15 and IL-2 was detected. Data are means ± s.e.m.

of mature memory T cells[31], and subsequently modified by the cytokine environment within synovial tissue, particularly IL-15. Our finding that IL-15-induced CD69 expression was primarily restricted to CD45RO$^+$ T cells is compatible with these observations, as well as with a recent report comparing the effect of IL-15 on naive and memory T-cell CD69 expression[32]. Despite extensive efforts to determine the synovial T-cell repertoire, no consensus has emerged for oligoclonal T-cell receptor (TCR) *Vβ* gene expression between RA patient cohorts, although some amino acid conservation of CDR3 regions has been reported, raising the possibility that some synovial T cells are antigen driven[33]. The majority of synovial T cells, however, are polyclonal. T cell-contact-mediated macrophage activation, driven by IL-15, is therefore consistent with the recognized phenotype and functional profile of synovial T cells. It provides a pathologic role, but implies no local antigen recognition and requires no cytokine secretion, consistent with the polyclonality of synovial T lymphocytes, and their relative absence of cytokine expression.

Cell contact is required for IL-15-induced TNF-α synthesis. Following chemokine-induced polarization, T lymphoblasts redistribute adhesion molecules, such as ICAM-1 or ICAM-3, to the tips of uropods, and IL-15 has recently been shown to exhibit similar properties[34]. However, since we were unable to induce macrophage activation by T cells maintained with the T-cell chemotactic factors, IL-8 or MIP-1α, it seems probable that IL-15 must exert effects beyond ligand redistribution. Increased CD69 expression occurs *in vivo* on RA synovial T cells[28,30] and has been reported in multiple sclerosis and chronic active hepatitis at sites of inflammation[35,36]. CD69 appears following TCR–antigen interaction, although IL-2 alone may upregulate expression on NK cells and CD45RO$^+$ lymphocytes[37]. Our data show that IL-15 shares this activity with IL-2 and is therefore likely to account for the continued expression of CD69 characteristic of synovial T cells, in the relative absence of IL-2 in synovial tissue[38]. Although CD69 has previously been shown to mediate mitogen-induced T-cell/macrophage contact[39], our data demonstrate that it might subserve this function in RA. Both IL-2 and IL-15 upregulated CD69 expression equally in PB lymphocytes, but IL-2 was significantly less efficient than IL-15 at inducing T cell-dependent macrophage activation, either in PB cells, or in synovial cells. This implies that other receptors which are involved in cell contact are preferentially upregulated by IL-15, compared with IL-2, and also indicates that the precise combination of surface markers present is likely to be of critical importance. The widespread expression of CD69 on other circulating cells, such as platelets[39], requires that such additional levels of regulation be present.

Interleukin-15 and IL-2 are functionally distinct. The bioactivities described thus far for IL-15 have been broadly similar to those of IL-2 (ref. 13). We found similar SF T-cell proliferation but differential direct TNF-α production to IL-15 and IL-2. In combination with the divergent effects of IL-15 and IL-2 in generating PB and SF T cell-induced macrophage activation, we have shown, in an immunopathologic setting that IL-15 exhibits effects distinct from IL-2 on the same target cell population. This has implications for IL-15 receptor (IL-15R) expression within the synovial membrane. It is possible that IL-15R α-chain binding modifies the signal generated by IL-2Rβ or common γ chain occupancy, although the α-chain has no apparent direct role in signal transduction[15,16]. The distribution of IL-15R α-chain compared with IL-2R α-chain in the synovium, however, is currently unknown, and discordant expression may partially explain our data. Recently, however, a novel receptor for IL-15 has been described on mast cells[40], and it remains possible that expression of this or related, as yet undescribed, receptors may explain the divergent functional profile for IL-15 and IL-2 in the synovial T-cell population.

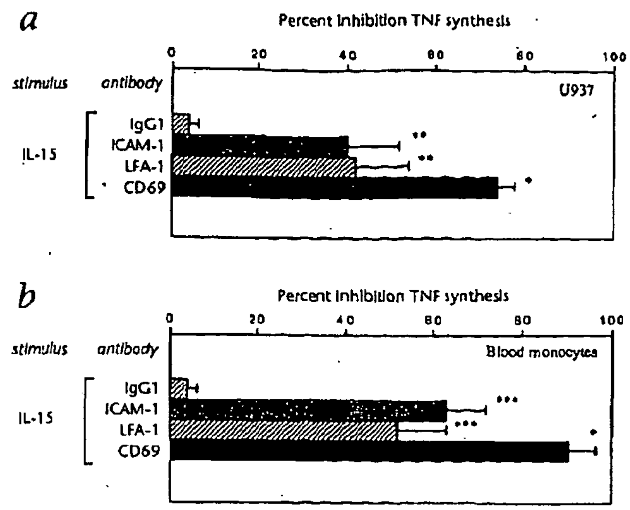


Fig. 7 Inhibition of cell-contact induced TNF-α synthesis by antibodies to cell-surface markers. PFA-fixed IL-15-activated PB T cells were cultured with (*a*) U937 cells, or (*b*) PB monocytes. Preincubation of the T cells with neutralizing antibodies to CD69, LFA-1 or ICAM-1 significantly inhibited TNF-α synthesis compared with that from IgG1 control antibody-treated cells (*P < 0.001, **P < 0.03, ***P < 0.01). Data are means ± s.e.m. from four separate experiments. Percent inhibition was calculated as follows:

$$100 - \left(\frac{\text{TNF-}\alpha\text{ synthesis with neutralizing antibody present}}{\text{TNF-}\alpha\text{ synthesis without neutralizing antibody present}} \times 100\right)$$

Conclusions. In the presence of a clearly defined antigen, non-antigen driven processes within the synovial membrane may well offer the best targets for therapeutic intervention.[1] We hypothesize that macrophage- or fibroblast-derived IL-15 recruits and further expands circulating memory T cells in the synovial membrane. The expansion of memory T cells by IL-15 is not incompatible with the two-signal theory of T-cell activation. Under the continuing influence of IL-15, newly arrived memory T cells within the synovial membrane can upregulate TNF-α production by macrophages, predominantly through cell-membrane contact. This may generate a positive feedback loop, whereby IL-15 produced by activated macrophages maintains T cell-induced synthesis of TNF-α, which can further activate macrophages to produce IL-15. This hypothesis implies an excessive activation of IL-15 synthesis and/or a breakdown of normal control of IL-15 regulation in RA. Further efforts are now required to characterize other cell-surface molecules involved in T-cell/macrophage contact and to establish which factors upregulate IL-15 production, thereby generating further rational targets for novel therapy.

Methods

Patients. Peripheral blood (PB) and synovial fluid (SF) samples were col-

lected from 25 RA patients who satisfied the American College of Rheumatology 1987 criteria[11], with mean age 62.4 years (range 24–83) and positive rheumatoid factor in 90%. All patients received disease-modifying drug therapy but none received intra-articular corticosteroid within 3 months of sampling. Buffy coats were obtained from normal volunteer donors to the Blood Transfusion Service (Carluke, UK). IL-15 was detected in 8 of the synovial fluid samples and used in Figures 1 and 2. IL-15 was assayed by ELISA using paired murine monoclonal antibodies (BAM247 & MAB647, R&D Systems, Abingdon, UK). Mean IL-15 concentration in the positive synovial fluid samples was 526 pg/ml ± 58 pg/ml (range 50–1134 pg/ml). Removal of rheumatoid factor from synovial fluid using γ-globulin coated polystyrene beads (Rapitx RF, Behring, Milton Keynes, UK) did not significantly alter the results obtained in this ELISA.

Cell purification and culture. Cultures were maintained in RPMI, with 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal calf serum (FCS) (all Gibco BRL, Paisley, UK). PB or SF mononuclear cell fractions were obtained by density gradient centrifugation (Lymphoprep, Nycomed Pharma, Oslo, Norway). T lymphocytes were enriched by successive 1-h, then 16-h adherence at 37 °C. The resulting populations were >92% CD3⁺, <2% CD14⁺ <3% CD19⁺ for PB, and >90% CD3⁺, <3% CD68⁺, <2% CD19⁺ for SF by fluorescence-activated cell sorting (FACS) analysis (Becton Dickinson, Oxford, UK) or Cytoprep analysis (antibodies from Dako, High Wycombe, UK), respectively.

T-cell proliferation and direct TNF-α production. Enriched T cells from PB/SF were cultured in triplicate at 2×10^5 cells/well in 96-well U-bottom plates (Nunc, Roskilde, Denmark) in the presence or absence of rIL-15 (gift from D. Cosman, Immunex Corp., Seattle, WA), rIL-2 (R&D Systems, Oxon, UK), or 1 µg/ml phytohemagglutinin (PHA, Murex Diagnostics Ltd., Dartford, UK). [^{3}H]Thymidine (1 µCi/well, Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK) was added 6 h before harvesting (Micromate 196, Matrix 96 direct beta counter, Packard, Pangbourne, UK). Data were presented as stimulation indices: (mean c.p.m. of test culture)/(mean c.p.m. of medium control). Medium controls ranged from 100 to 2993 c.p.m. TNF-α synthesis was measured in identical parallel culture supernatants harvested after 24, 48 or 72 h and stored at –70 °C.

Cell-contact experiments. Cell-contact experiments were performed as previously described[23]. PB T cells were cultured for 72 h in 5% FCS, in the presence of medium alone, rIL-15, rIL-2, rIL-8 (R&D Systems), or rMIP-1α (gift of G.J. Graham, CRC Beatson Institute, Glasgow, UK) all at 100 ng/ml or with a combination of 1 µg/ml PHA/1 nM phorbol myristate acetate (PMA, Sigma Chemicals, Poole, UK). Cells were washed three times then fixed for 2 h in 1% paraformaldehyde in PBS (Sigma) at 4 °C with gentle agitation. SF T cells similarly prepared were cultured with or without stimulus for only 16 h before fixation. Fixed cells were washed three times, resuspended to 4×10^6 cells/ml in RPMI, then immediately added to 5×10^5 cells/ml U937 cells, PB monocytes or synovial macrophages/synoviocytes in 10% FCS. After 48 h, supernatants were harvested and stored at –70 °C for TNF-α estimation. Preliminary experiments established this optimal cytokine concentration and cell ratio for TNF-α production. PB monocytes and macrophage/synoviocyte cultures were prepared by retaining adherent cells during the T-cell enrichment process. PB monocyte purity was >94% CD14⁺ after 24 h, with <2% CD3⁺, <2% CD19⁺ by FACS analysis (Becton Dickinson). Synovial adherent cells were >79% CD68⁺, <4% CD3⁺ by Cytoprep analysis.

Double-chamber culture. PB T cells were stimulated for 72 h as described, then added without fixation at 4×10^6 cells/ml to U937 cells at 5×10^5 cells/ml. Identical parallel cultures were established in which PB T cells were separated from U937 cells by a culture insert (Falcon, Becton Dickinson). Supernatants were harvested after 48 h for TNF-α estimation.

Inhibition of TNF-α synthesis by antibody. Antibodies against CD69 (Becton Dickinson), LFA-1 (CD11a), or ICAM-1 (CD54) (gifts from N. Hogg, Imperial Cancer Research, London, UK) or IgG1 of irrelevant specificity (Dako) were incubated at 5 µg/ml with PFA-fixed T cells for 30 min at room temperature. Cells were washed three times before addition to U937 cells or PB monocytes as before. Increasing the anti-LFA-1 or anti-ICAM-1 antibodies to 10 or 25 µg/ml or anti-CD69 antibody to 50 µg/ml did not lead to further inhibition of TNF-α production (data not shown). To minimize monocyte FcγR engagement by cell-bound antibody, we added 5 µg/ml human IgG (Sigma) to these cocultures. TNF-α synthesis after 48 h was estimated by ELISA.

Tumor necrosis factor-α measurement. This was detected by ELISA using paired murine monoclonal anti-human TNF-α antibodies (MAb1 and MAb11, PharMingen). Standard rTNF-α was a gift from G.A. Adolf, Bender Wien, Austria. Sensitivity was <10 pg/ml.

Analysis by FACS of whole-blood culture. Heparinized blood was diluted 1:5 in Iscove's medium (Gibco BRL), and triplicate cultures were established to which were added 100 ng/ml rIL-15, rIL-2 or 1 µg/ml PHA. Cells were harvested at time intervals from 1 h to 72 h for double-staining using the following markers: CD3 (FITC and PE), CD56 (PE), CD16 (PE), CD69 (FITC and PE), CD45RA (FITC), CD45RO (PE), CD19 (FITC) (all Becton Dickinson), CD11a, CD54, both detected with FITC-conjugated F(ab')$_2$ rabbit anti-mouse IgG (Dako). Antibodies were added to 100 µl of whole-blood culture at 4 °C for 30 min. Secondary antibody was added if required after 15 min. Negative control antibodies of appropriate isotype and conjugate (Dako) were similarly processed. Leukocytes were recovered using FACS lysing solution (Becton Dickinson) and analyzed on a FACScan (Becton Dickinson). Gates were set for lymphocytes using forward and side light scatter parameters, and the percentage of single FITC-conjugated, single PE-conjugated or double-positive cells in this region was generated using Lysis II software (Becton Dickinson).

Statistical analysis. Comparison between groups was by Mann-Whitney test. Paired samples were compared using a *t*-test or Wilcoxon matched pairs signed rank sum test.

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A potential role for CD69 in thymocyte emigration

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Abstract

The early activation marker, CD69, is transiently expressed on activated mature T cells and on thymocytes that are undergoing positive or negative selection in the thymus. CD69 is a member of the NK gene complex family of C-type lectin-like signaling receptors; however, its function is unknown. In this report, we describe the characterization of mice that constitutively express high levels of surface CD69 on immature and mature T cells throughout development. Constitutive surface expression of CD69 did not affect T cell maturation, signaling through the TCR or thymocyte selection. However, phenotypically and functionally mature thymocytes accumulated in the medulla of CD69 transgenic mice and failed to be exported from the thymus. The retention of mature thymocytes correlated with transgene dose and CD69 surface levels. These results identify a potential role for CD69 in controlling thymocyte export, and suggest that the transient expression of CD69 on thymocytes and T cells may function to regulate thymocyte and T cell trafficking.

Introduction

Lymphocyte migration is known to play an important role in regulating the localization and orchestration of immune responses. Although less well defined, the processes that regulate trafficking of lymphoid precursors to and within sites that support their development, and that mediate the subsequent emigration of mature cells to the periphery, are also critical for the establishment of cellular immunity. T lymphocyte precursors that originate in the fetal liver or bone marrow migrate to the thymus and subsequently undergo a complex differentiation process that includes positive and negative selection, before emerging as $CD4^+$ or $CD8^+$ single-positive (SP) T cells. As thymocytes progress through these developmental stages, they migrate from the subcapsular region of the thymus to the cortex and then to the medulla (1). At the conclusion of this maturation process, functionally mature thymocytes exit the thymus and seed the peripheral lymphoid tissues.

Results from several recent studies indicate that newly generated SP thymocytes undergo several additional maturation steps before being exported from the thymus (2,3). In addition, fully mature SP thymocytes also display a phenotype that resembles that of recent thymic emigrants (RTE) which is distinct from most other medullary thymocytes (4,5). The mechanisms that control thymocyte emigration and the molecules involved in this process remain ill-defined. A pertussis toxin-sensitive pathway, therefore likely involving G_i-proteins, has been implicated in regulating thymocyte emigration (6,7). Chemokine receptors, which have been shown to regulate migration of mature lymphocytes and which signal through G-protein-mediated pathways, are therefore leading candidates for controlling thymocyte export (8). Adhesion molecules and their signaling pathways are also likely to play important roles in thymocyte emigration (9).

The early activation marker, CD69, is a member of the NK cell gene complex family of C-type lectin-like signal-transducing receptors. CD69 is a type II transmembrane glycoprotein that contains a C-type lectin-binding extracellular domain (10–13). Although CD69 is expressed by a wide range of hematopoietic lineages, its function and ligand remain unknown. The timing and pattern of CD69 induction is similar in all cells that express this protein and is characterized by

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rapid but transient surface expression. In T cells, CD69 is upregulated very early after TCR triggering and wanes soon after stimulation withdrawal (14–17). In the thymus, immature $CD4^+CD8^+$ [double-positive (DP)] thymocytes that are undergoing positive or negative selection also transiently express CD69 (18–21). However, the lack of a thymocyte or T cell phenotype in $CD69^{-/-}$ mice suggests that it does not play a critical role in T cell maturation (22).

To examine the function of CD69 in T cell development, we generated CD69 transgenic (Tg) mice that constitutively express CD69 at all stages of T cell development. We show here that overexpression of CD69 does not impair thymocyte development, but results in accumulation of functionally mature SP thymocytes in the thymus and a paucity of T cells in the periphery. Our results suggest a role for CD69 in controlling the export of mature cells from the thymus and indicate that transient activation-induced CD69 surface expression may be important for regulating T cell trafficking.

Methods

Reagents and antibodies

Fluorochrome-conjugated anti-CD3, -CD4, -CD8, -CD24, -CD69, -CD62L, -β_7 integrin, and biotinylated anti-CD8 and -CD45R (B220) mAb were purchased from PharMingen (San Diego, CA). 5-Bromodeoxyuridine (BrdU) was purchased from Sigma-Aldrich (St Louis, MO). CFSE was purchased from Molecular Probes (Eugene, OR). Allophycocyanin-conjugated CD8 for confocal staining was purchased from Caltag (Burlingame, CA). Alexa Fluor 488-conjugated anti-CD4 was prepared by labeling purified anti-CD4 mAb (PharMingen) using the Alexa Fluor 488 protein labeling kit (Molecular Probes) according to the manufacturer's instructions.

Mice

CD69 Tg mice were established and maintained in our animal facility under specific pathogen-free conditions. The transgene construct was generated by cloning the CD69 coding region into a vector containing the human CD2 promotor and enhancer. The resulting construct (huCD2–CD69) was linearized and injected into B6 zygotes (23). C57BL/6 and $Rag\text{-}2^{-/-}$ mice were purchased from Taconic (Germantown, NY). TCR Tg mice used in these studies included H-Y and AND TCR Tg mice. H-Y mice express an MHC class I-restricted TCR for the male antigen H-Y (24) and AND mice express an MHC class II-restricted TCR specific for pigeon cytochrome *c* (25). All TCR Tg mice were maintained in the $H\text{-}2D^b$ background.

Flow cytometry

Cells (1×10^6/sample) isolated from thymus, lymph nodes or spleens were suspended in 50 μl of FACS buffer (1 × HBSS, 0.1% BSA and 0.01% sodium azide) with a cocktail of FITC-, phycoerythrin-, Quantum Red-, Per-CP- or allophycocyanin-conjugated antibodies. The mixtures were incubated at 4°C for 1 h. Unbound antibodies were washed out and cells were resuspended in FACS buffer, and then analyzed by flow cytometry using a FACScan or FACSCalibur instrument (Becton Dickinson, San Jose, CA).

BrdU labeling of thymocytes

BrdU (0.8 mg/ml) was administrated continuously to mice in drinking water. Thymocytes were isolated from treated mice at various time points, and 2×10^6 cells/sample were stained with fluorochrome-conjugated anti-CD4 and -CD8 mAb. After washing out the unbound antibodies, cells were fixed with ethanol and paraformaldehyde (PFA). After DNase I treatment of fixed cells, intracellular staining was performed using FITC-conjugated anti-BrdU antibody (PharMingen). Cells were washed and suspended in PBS for analysis by flow cytometry.

Purification and adoptive transfer of CD4 SP thymocytes

Thymocytes were suspended in 4×10^7/ml PBS containing 1% BSA, and incubated with biotinylated anti-CD8 and -B220 antibodies. After incubation on ice for 15 min, unbound antibodies were washed out with PBS and 10 μl of streptavidin–beads (Miltenyi Biotec, Auburn, CA) was added. After another 15-min incubation, cells were passed through a magnetic separation column (Miltenyi Biotec). The flow-throughs were collected, and the purity of each fraction was checked by staining with CD4 and CD8 antibodies. CD4 SP cells in purified populations were typically >80% from non-Tg mice and >90% from CD69 Tg mice. Purified CD4 SP thymocytes were suspended in PBS (2×10^7/ml) and i.v. injected into $Rag\text{-}2^{-/-}$ or C57BL/6 mice. In indicated experiments, purified cells were resuspended in PBS (1×10^7/ml) and stained with 1 μM CFSE for 10 min at 37°C before transfer. Cells were washed twice with PBS and re-suspended in PBS for injection. Cells from indicated organs of recipients were isolated and analyzed with antibody staining at different time points after transfer. Recoveries were calculated by multiplying total cell number by the percentage of CD4 SP or CD4 SP $CFSE^+$ cells in each organ.

Proliferation assay

Single-cell suspensions were prepared from thymi in RPMI plus 10% FCS. CD4 SP thymocytes were enriched by panning with CD8, B220 and Mac-1 antibodies on rabbit anti-mouse IgG-coated plates, followed by a positive selection by magnetic separation using CD4–biotin/streptavidin microbeads in the MACS system (Miltenyi Biotec) as indicated above. Accessory cells and antigen-presenting cells (APC) were prepared from spleen cell suspensions from B10.A mice. APC were depleted of T cells with anti-Thy 1.2 + C′ and irradiated with 3000 rad. Then 1×10^5 responder T cells were combined with 5×10^5 accessory cells in flat-bottom 96-well plates in the presence or absence of the indicated stimulants. To determine the dose response, a constant number of APC (5×10^5) was combined with 2-fold dilutions of responder T cells. A peptide of pigeon cytochrome *c* (fragment 81–104) was synthesized in the FDA Core Facility and added to culture at the indicated concentrations. Following stimulation for 48 h, cells were pulsed for 12 h with 1μCi [^{3}H]thymidine and harvested. Antibodies used for panning, including anti-CD8 (2.43), -B220 (6B2) and -MAC-1 (M1/70), were purified with Protein G from tissue culture supernatant generated from B cell hybridomas grown in an Artificial Capillary System (Cellco, Germantown, MD).

Confocal microscopy

Thymi were fixed with 4% PFA for 10 min before being embedded and frozen in OCT compound (Sakura Finetec, Torrance, CA). Cryostat sections (10 μm) were prepared (Histoserv, Gaithersburg, MD). Sections were washed in PBS, fixed with 4% PFA and quenched with 50 mM NH_4Cl before staining. Sections were incubated with Alexa Fluor 488-conjugated anti-CD4 and allophycocyanin-conjugated CD8 at 4°C overnight in the dark. Sections were washed, mounted with Prolong Antifade reagent (Molecular Probes) and dried overnight at room temperature with drierite, and then analyzed by confocal microscopy.

Results

Phenotype of CD69 Tg mice

Four huCD2–CD69 Tg (CD69 Tg) founder lines were generated by zygote micro-injection and analyzed. To assess CD69 transgene expression, we first examined CD69 surface levels on thymocyte subsets by flow cytometry. In non-Tg mice, CD69 is expressed on the surface of a minor population of $CD4^+CD8^+$ [double-positive (DP)] and 'transitional' $CD4^+CD8^-$ (CD4 SP) and $CD4^-CD8^+$ (CD8 SP) thymocytes (2,18). In contrast, in CD69 Tg mice, surface expression of CD69 was observed on all thymocyte subsets (Fig. 1A and C, and data not shown). Moreover, CD69 surface levels paralleled transgene copy number (data not shown). Representative founder lines containing either a low (line #2005) or high (line #2028) transgene copy number were chosen for further investigation.

Constitutive surface expression of CD69 had no apparent effect on early thymocyte development as assessed by the normal distribution of double-negative (DN) thymocyte subsets (based on staining with CD25 and CD44; data not shown) and the presence of normal numbers of DP thymocytes in CD69 Tg mice. However, mice from all CD69 Tg founder lines contained higher percentages and numbers of CD4 SP and CD8 SP thymocytes relative to non-Tg littermates (Fig. 1B). The increased number of SP thymocytes in CD69 Tg mice was consistently observed and was accompanied by a concomitant decrease in the percentage, but not the number, of precursor DP thymocytes (Fig. 1B).

Surprisingly, examination of peripheral lymphoid organs revealed that the accumulation of SP thymocytes in CD69 Tg mice also correlated with a decrease in the number of mature peripheral CD4 SP and CD8 SP T cells (Fig. 1B). The reduction in peripheral T cell numbers also paralleled transgene copy number and was observed in all secondary lymphoid organs examined including lymph nodes, spleen and Peyer's patches (Fig. 1B and data not shown). Analysis of the phenotype of the few T cells present in lymph nodes of the representative high-copy-number line, #2028, revealed that only ~50–60% of the cells were $CD69^+$ (Fig. 1D, left column). In addition, most T cells were $CD62L^{low}$ $CD44^{high}$, suggesting that they may have undergone homeostatic proliferation (data not shown). Similar to αβ-lineage cells, the number of $\gamma\delta TCR^+$ thymocytes was increased, but the number of peripheral γδ T cells was decreased in high-copy-number CD69 Tg mice (data not shown).

In contrast to the results obtained with the high-copy-number Tg lines, normal or only slightly reduced numbers of peripheral T cells were found in low-copy-number CD69 Tg mice. As in non-Tg mice, these cells were predominantly $CD69^-$ (Fig. 1D, middle column). Analysis of T cells from the low-copy-number CD69 Tg lines by Southern blotting confirmed that the transgene was still present; however, Tg CD69 mRNA levels were reduced relative to those of total thymocytes from the same mice (data not shown). These data suggest that the absence of CD69 surface expression on most peripheral T cells in low-copy-number Tg mice is most likely due to a reduction in transgene mRNA expression. Indeed, previous data indicate that huCD2-mediated transgene expression decreases in mature T cells relative to immature thymocytes (26). Consistent with this interpretation, we observed that although CD69 surface expression was elevated on DP thymocytes from line #2005 Tg mice, it was similar to that of non-Tg mice on SP thymocytes (Fig. 1C, middle column). This suggested that the reduction in CD69 mRNA levels and CD69 surface expression occurred at the SP stage. The reduction in CD69 expression in SP thymocytes relative to DP thymocytes was also seen in the high-copy-number Tg lines, although most SP thymocytes still expressed CD69 (Fig. 1C, left column; data not shown).

Kinetics of thymocyte development in CD69 Tg mice

To determine if the increase in SP thymocytes in CD69 Tg mice was due to a faster maturation rate, we examined the kinetics of thymocyte development by analyzing thymocyte subsets following continuous BrdU oral administration. BrdU is incorporated into proliferating cells, which in the thymus are predominantly late DN thymocytes, and these cells remain $BrdU^+$ during subsequent maturation stages (27,28). As shown in Fig. 2(left panels), the kinetics of DP thymocyte labeling were similar in non-Tg and CD69 Tg mice as assessed by the absolute number and percentage of $BrdU^+$ DP thymocytes observed at different time points. Similar numbers of $BrdU^+$ CD4 SP thymocytes were also detected in CD69 Tg mice and non-Tg littermates, indicating that SP thymocytes are generated at the same frequency in these mice (Fig. 2A, right panel). However, the percentage of $BrdU^+$ cells among all CD4 SP thymocytes was consistently lower in CD69 Tg mice relative to non-Tg littermates (Fig. 2B, right panel). The time required to label 50% of all CD4 SP thymocytes ($t_{1/2}$) was ~6 days in non-Tg mice, consistent with previous reports indicating that the average intrathymic 'lifespan' of SP thymocytes is 12 days (29). However, in CD69 Tg mice, the $t_{1/2}$ was prolonged to at least 12–14 days, indicating that SP thymocytes remain in the thymus for a much longer time than in non-Tg littermates (Fig. 2).

Thymocyte selection is unaffected in CD69 Tg mice

DP thymocytes that receive activating signals in the thymus by interacting with positively or negatively selecting ligands transiently express CD69 (18–21). To determine if the increase in SP thymocytes in CD69 Tg mice was due to an alteration in thymocyte selection, we bred TCR transgenes into the CD69 Tg background and examined the efficiency of thymocyte selection. The MHC class I-restricted TCR transgene, H-Y, promotes positive selection of large numbers of CD8 SP

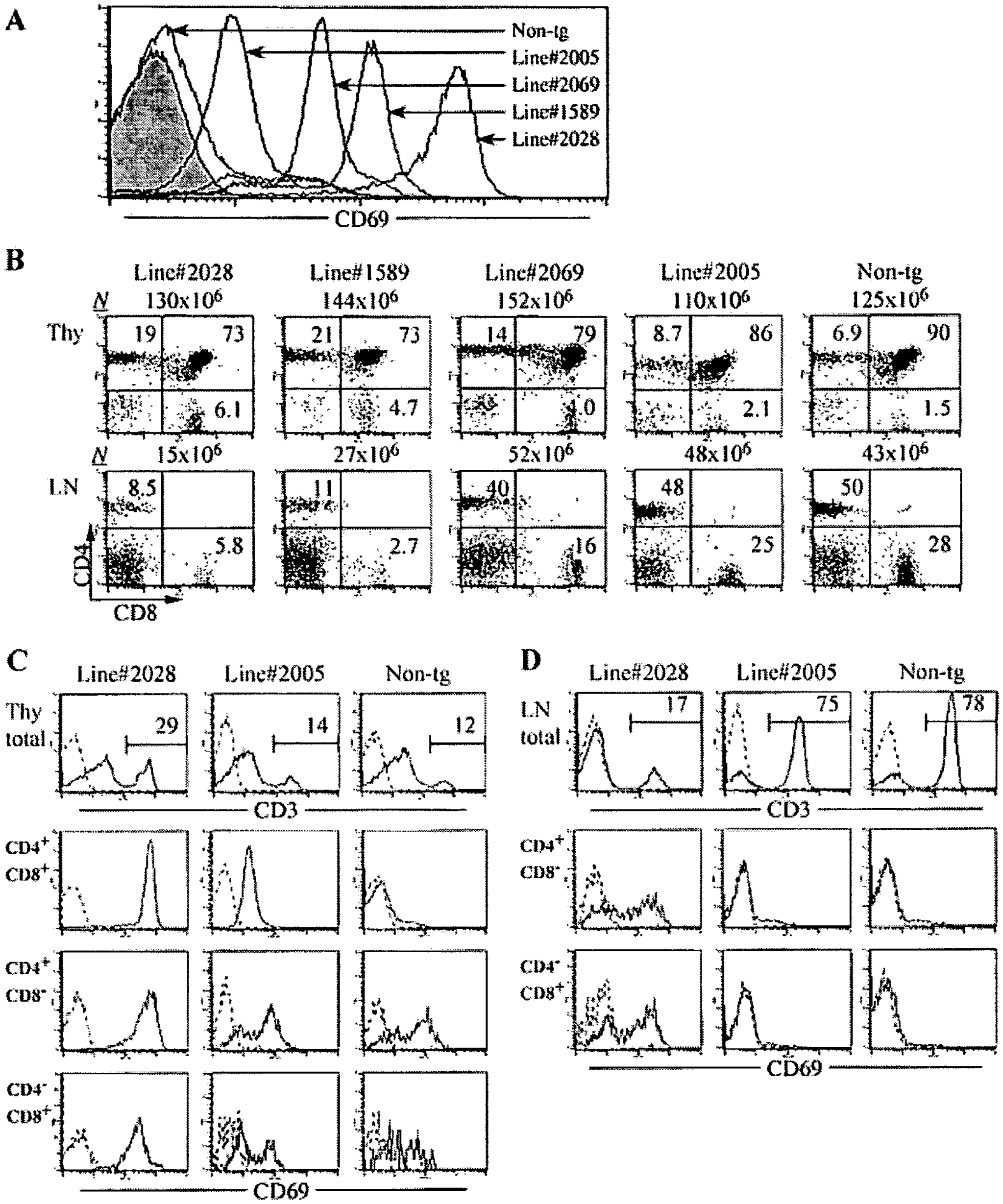

Fig. 1. Phenotype of CD69 Tg mice. Thymocytes and lymph node cells isolated from CD69 Tg and non-Tg mice were stained with anti-CD4, anti-CD8 and either control, anti-CD3 or anti-CD69 mAb, and analyzed by flow cytometry. (A) CD69 expression levels on total thymocytes from four founder lines and a non-Tg littermate are shown. (B) CD4 versus CD8 staining profiles of thymocytes (Thy) and lymph node (LN) cells, with representative total cell numbers (*N*) and percentages of each population shown. CD3 expression levels in total thymocytes (C) and lymph node cells (D), as well as CD69 expression levels on DP (CD4$^+$CD8$^+$) and SP (CD4$^+$CD8$^-$ and CD4$^-$CD8$^+$) thymocytes, and SP (CD4$^+$CD8$^-$, CD4$^-$CD8$^+$) lymph node T cells are shown.

thymocytes in female mice and strong negative selection of thymocytes in male mice (30,31). Comparison of H-Y TCR Tg × CD69 Tg and H-Y TCR Tg male mice revealed no difference in the efficiency of negative selection (Fig. 3B). In addition, accumulation of CD8^{low}CD4$^-$ cells in the thymus and their paucity in the periphery was observed in H-Y TCR Tg × CD69 Tg just as in non-TCR Tg × CD69 Tg mice (Fig. 3B). In order to distinguish the effect of constitutive CD69 expression on thymocyte positive selection from accumulation of SP thymocytes, we chose to analyze the low-copy-number CD69 Tg line, #2005, to assess positive selection. In this line, CD69 expression is elevated on all DP thymocytes, but only a slight accumulation of SP thymocytes is observed in non-TCR Tg mice (Fig. 1B). Examination of H-Y TCR Tg × CD69 Tg females revealed no significant difference in the efficiency of positive selection relative to H-Y TCR Tg littermates (Fig. 3A). We also observed no difference in positive selection when the MHC class II-restricted TCR transgene AND was tested (data not shown). Finally, the extent of activation-induced death of DP thymocytes in response to CD3 plus CD28 stimulation was

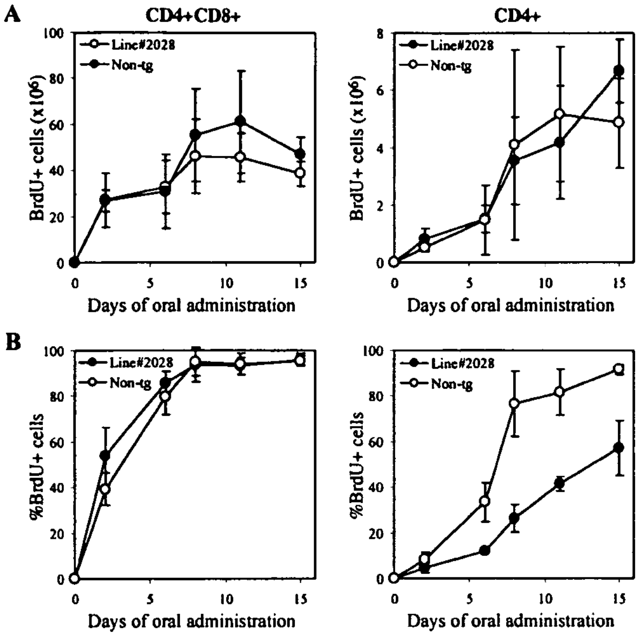

Fig. 2. Kinetics of thymocyte development in CD69 Tg mice. CD69 Tg mice (6–10 weeks old, line #2028) and their non-Tg littermates were continuously administrated BrdU (0.8 mg/ml) through their drinking water. Mice were sacrificed at different time points, and the number and percentage of BrdU$^+$ thymocytes was determined. Numbers (A) and percentages (B) of BrdU$^+$ cell in the CD4$^+$CD8$^+$ (left panels) and CD4$^+$CD8$^-$ (CD4$^+$) (right panels) compartments are shown. At least three mice were used for each time point.

similar in CD69 Tg mice and non-Tg littermates (data not shown).

Accumulation of mature SP thymocytes in high-copy-number CD69 Tg mice is due to failure of thymocyte emigration

To explore further the reason for the accumulation of SP thymocytes in high-copy-number CD69 Tg mice, we analyzed thymocyte profiles from mice beginning at birth when the first wave of mature SP cells is observed in the thymus. There was no significant difference in the percentage of SP thymocytes between CD69 Tg mice and non-Tg littermates on day 1, again indicating that the rate of SP thymocyte formation was not increased in CD69 Tg mice relative to non-Tg littermates (Fig. 4A). However, increased numbers and percentages of SP thymocytes were observed in CD69 Tg mice by day 5 and were consistently seen thereafter (Fig. 4A). SP T cells were detectable in spleens of non-Tg mice on day 5 and on all subsequent days, whereas peripheral T cells were first detected in low numbers only on day 10 or later in CD69 Tg mice (Fig. 4B). These results indicated that constitutive expression of CD69 either inhibits the complete maturation of SP thymocytes or inhibits export of SP thymocytes from the thymus.

Development of newly generated SP thymocytes into functionally mature T cells is a multi-step process that occurs as these cells migrate through the medulla (2,3). Thus, the accumulation of SP thymocytes in CD69 Tg mice could be due to failure of these cells to complete the maturation process.

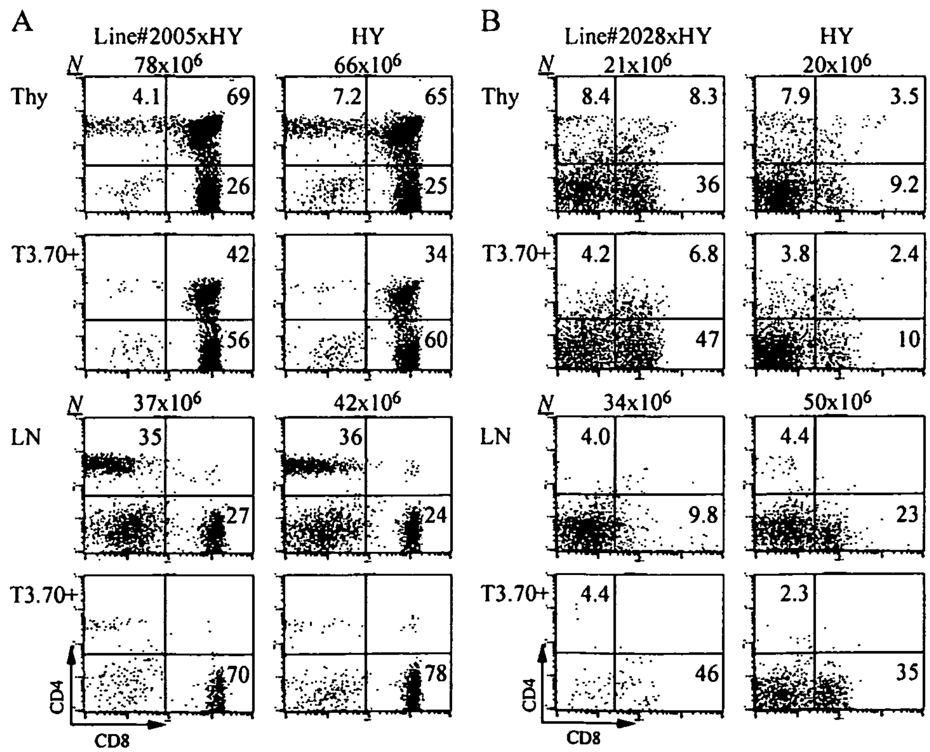

Fig. 3. Positive and negative selection in CD69 Tg mice. CD69 Tg mice were mated to HY-TCR Tg mice. Thymocytes and lymph node cells, isolated from double-Tg or HY-TCR Tg female (A) and male (B) mice, were stained with mAb and analyzed by flow cytometry. Total cells numbers (*N*), and CD4 and CD8 profiles with percentage of each subset are shown.

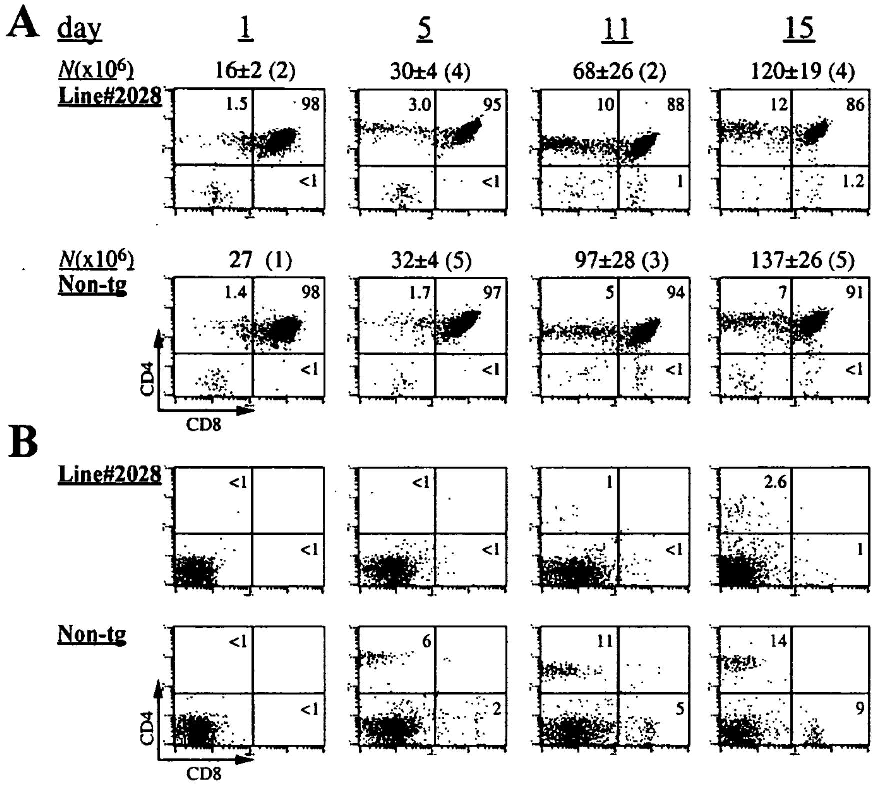

Fig. 4. Effect of CD69 transgene on postnatal T cell development. CD69 Tg mice (line #2028) and non-Tg littermates were sacrificed 1, 5, 11 and 15 days after birth, and their thymocytes (A) and splenocytes (B) were analyzed by flow cytometry. Numbers indicate the percentage of cells in each compartment. Average thymocyte numbers (*N*) and numbers of mice analyzed are shown.

The phenotype of fully mature SP thymocytes has been previously established by examining the phenotype of RTE (4). RTE display phenotypes distinct from most other medullary thymocytes: CD4 SP RTE are CD24^{low}, CD62L^{high} β_7 integrin^{int} whereas CD8 SP RTE are CD24^{low}, CD62L^{high}, CD45RB^{high} β_7 integrin^{high}. Analysis of the percentage of RTE-phenotype CD4 SP and CD8 SP thymocytes revealed an ~2- to 3-fold increase in these subsets in CD69 Tg mice relative to non-Tg littermates (Fig. 5A). These results correlate with the 2- to 3-fold increase in total SP thymocyte subsets (Fig. 1B), indicating that most of the accumulated SP thymocytes in CD69 Tg mice are phenotypically mature. To determine if the SP thymocytes in CD69 Tg mice were functionally mature, CD4 SP cells from AND TCR Tg and AND TCR Tg × CD69 Tg mice were stimulated with varying concentrations of agonist peptide in the presence of APC. As shown in Fig. 5(B), the proliferative response of CD4 SP thymocytes was significantly greater with cells from AND TCR Tg × CD69 Tg mice relative to AND TCR Tg mice (Fig. 5B). To obtain an estimate of the number of functionally mature cells, the proliferation assay was repeated with serial dilutions of purified CD4 SP thymocytes from CD69 Tg and non-Tg mice. In agreement with the phenotypic analysis, these data indicated that AND TCR Tg × CD69 Tg mice contained approximately twice the number of functionally mature CD4 SP thymocytes as non-CD69 Tg/AND TCR Tg littermate controls (Fig. 5B, right panel). Taken together, these results indicate that constitutive expression of CD69 does not interfere with thymocyte development, but does inhibit the export of functionally mature SP thymocytes to the periphery.

Constitutive expression of CD69 does not affect mature T cell survival

In addition to a block in SP thymocyte emigration from the thymus, the paucity of T cells in lymph nodes and spleen of high-copy-number CD69 Tg mice could also be due to abnormal migration in the periphery or to the rapid death of thymic emigrants. To examine these possibilities, we adoptively transferred purified CD4 SP thymocytes from non-Tg or line #2028 CD69 Tg mice into Rag-2$^{-/-}$ recipients by i.v. tail injection. The recovery of donor cells from CD69 Tg mice 3 or 7 days after transfer in Rag-2$^{-/-}$ recipient spleens was comparable with that from non-Tg littermates (Fig. 6A). Few donor cells were detected in the thymus of Rag-2$^{-/-}$ recipients 7 days after transfer indicating that CD4 SP thymocytes from CD69 Tg mice do not preferentially return to the thymus (Fig. 6A). Analysis of donor CD4 SP thymocytes that had been pre-labeled with CFSE revealed that cells from CD69 Tg mice underwent homeostatic proliferation in Rag-2$^{-/-}$ recipients to a similar extent as non-Tg thymocytes, even though they remained CD69$^+$ (Fig. 6B). We also investigated the tissue localization of adoptively transferred CD4 SP thymocytes using C57BL/6 mice as recipients. No clear differences in migration to secondary lymphoid organs were detected between cells from CD69 Tg and non-Tg mice 24 h after

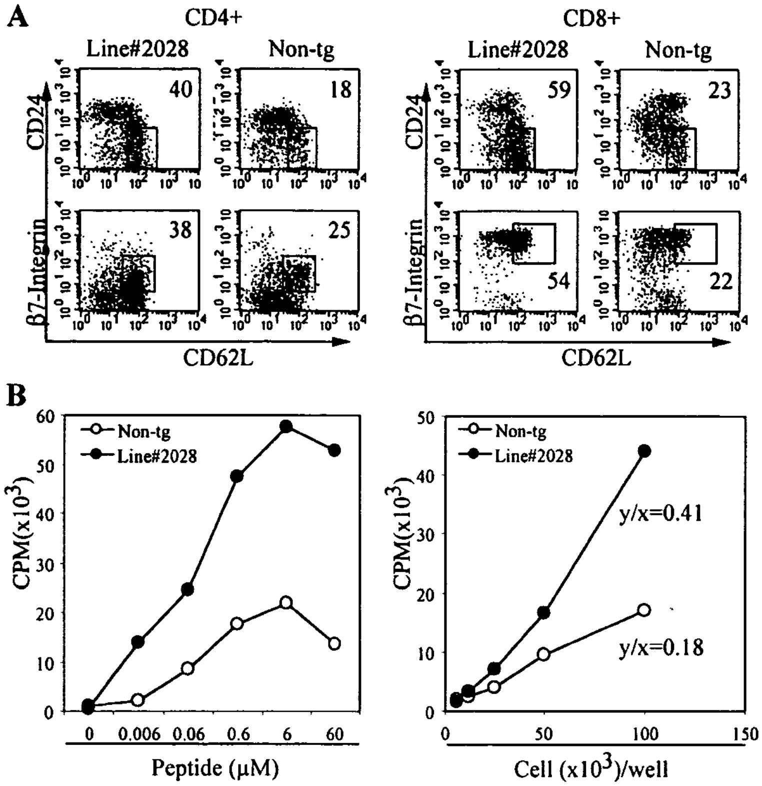

Fig. 5. Accumulation of phenotypically and functionally mature SP thymocytes in CD69 Tg mice. Thymocytes isolated from CD69 Tg (line #2028) mice and non-Tg littermates were stained with anti-CD62L, -β_7 integrin or -CD44, and anti-CD4 and -CD8 mAb, and analyzed by flow cytometry to determine percentages of cells with the RTE phenotype. Percentages (A) of $CD4^+CD8^-$ ($CD4^+$) (left panels) and $CD4^-CD8^+$ ($CD8^+$) (right panels) thymocytes with the RTE phenotype are shown. (B) Ability of $CD4^+$ thymocytes from CD69 Tg AND Tg and AND Tg mice to proliferate in response to pigeon cytochromic peptide. Peptide dose (left panel) and responder cell number (right panel) versus thymidine incorporation are shown. For the experiment shown in the left panel, 1×10^5 $CD4^+$ cells were used. For the experiment shown in the right panel, the peptide concentration used was 60 μM.

transfer (Fig. 6C). Interestingly, CD4 SP thymocytes from CD69 Tg mice did show a more rapid emigration from the blood to secondary lymphoid tissues (Fig. 6C). Taken together, these results indicate that constitutive CD69 surface expression does not influence T cell survival or result in abnormal compartmentalization of T cells within specific secondary lymphoid tissues.

Thymus architecture in CD69 Tg mice

Examination of H & E-stained sections of thymus from CD69 Tg mice revealed an enlarged medulla relative to non-Tg mice but normal cortical and medullary demarcation (Fig. 7A). Localization of the medulla was confirmed by staining thymus sections for thymic medullary epithelial antigen (data not shown). We also examined the intrathymic localization of DP and SP thymocytes by confocal microscopy. As in non-Tg mice, most CD4 SP (green) and CD8 SP (red) cells were located in the medulla, whereas DP (yellow) cells localized to the cortex (Fig. 7B). Consistent with the results obtained by H & E staining, higher cell densities were observed in the medulla of CD69 Tg thymi and these cells consisted almost entirely of SP thymocytes. CD69 Tg mice contained slightly increased numbers of apoptotic cells in the medulla (data not shown). In addition, 3–4% of *ex vivo* SP thymocytes were Annexin V^+ in CD69 Tg, compared to <1% in non-Tg mice (data not shown).

Discussion

CD69 is widely used as marker for lymphocyte activation (14,15,17,19–21). However, very little is known about its function in activated cells or its role in lymphocyte development, owing in part to its transient expression pattern. To explore the role of CD69 in T cell development, we generated Tg mice that constitutively express CD69 on developing T cells. The CD69 coding sequence was placed under the control of the human CD2 promotor and enhancer which directs T cell-specific, copy-number-dependent expression of transgenes in all thymocyte subsets and mature T cells (23). CD69 Tg mice displayed a transgene dose-dependent accumulation of mature SP thymocytes in the thymus and a concomitant reduction in the number of peripheral CD4 SP, CD8 SP and γδ T cells. Our results indicate that this phenotype is due to the failure of fully mature thymocytes to emigrate from the thymus. CD4 SP and CD8 SP thymocytes are produced with normal kinetics in CD69 Tg mice, and most of the SP cells

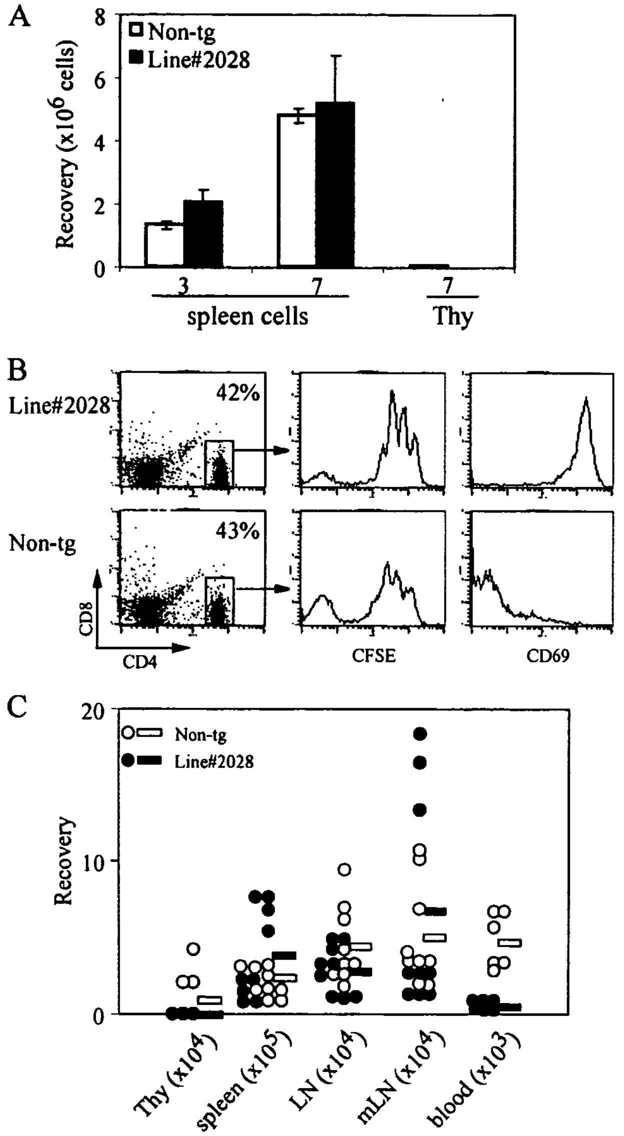

Fig. 6. Survival of CD4 SP thymocytes from CD69 Tg mice following adoptive transfer. CD4+ thymocytes from CD69 Tg (line #2028) mice and non-Tg littermates were purified by anti-CD8 and anti-B220 antibody depletion. Purified CD4+ T cells (3 × 10^6) without (A) or with (B and C) CFSE labeling were suspended in PBS and i.v. injected into non-irradiated Rag-2$^{-/-}$ (A and B) or C57BL/6 (C) mice. After 3 or 7 days, spleen cells and thymocytes of recipients were isolated, stained and analyzed by flow cytometry (A). Representative data of CFSE intensities and CD69 expression in CD4+ splenocytes from recipients 7 days after transfer are shown (B). Numbers of CFSE+ cells recovered from indicated organs after 24 h transfer are shown (C).

that accumulate in the thymus are phenotypically and functionally mature. The accumulation of SP thymocytes is not due to re-entry of exported cells, since adoptively transferred SP thymocytes that express high surface levels of CD69 do not preferentially home to the thymus. Moreover, the paucity of peripheral T cells in CD69 Tg mice cannot be attributed to rapid death of exported cells or to the failure of cells to migrate

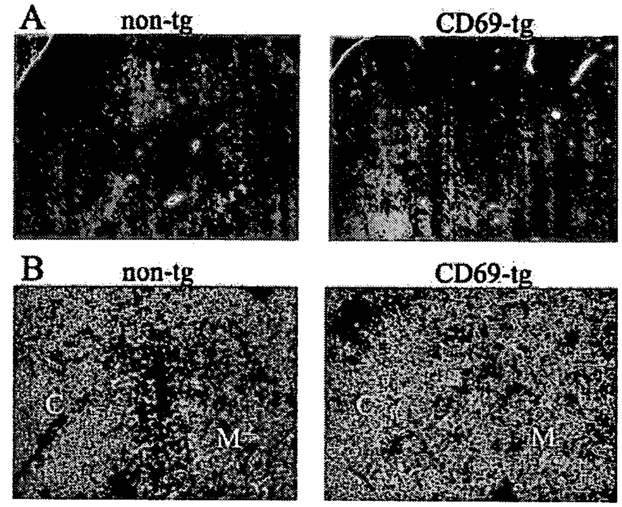

Fig. 7. Immunohistochemical analysis of thymi from CD69 Tg. Thymi from 6- to 8-week-old CD69 Tg mice (right panel) and non-Tg littermates (left panel) were harvested and frozen for staining. H & E staining was performed to examine thymic architecture (A, ×4 magnification). CD4 and CD8 staining was performed to localize thymocyte subsets and was analyzed by confocal microscopy (B, ×40 magnification). Green, CD4; red, CD8.

to and seed secondary lymphoid organs. Finally, constitutive expression of CD69 results in expansion of the medullary region of the thymus, presumably due to the accumulation of SP thymocytes, but does not cause alterations in thymic architecture or effect the normal pattern of thymocyte localization within the thymus.

Perhaps the simplest interpretation of the CD69 Tg phenotype is that overexpression of CD69 prevents emigration of SP thymocytes by binding to its ligand and trapping cells in the medulla. Although our data do not exclude this interpretation, several lines of evidence favor a more complex mechanism. First, although a number of other cell surface molecules have been overexpressed in thymocytes, none of these Tg mice exhibit a phenotype similar to that observed in CD69 Tg mice, including mice that overexpress another C-type lectin-like receptor, Ly49A (32). Second, we were unable to induce the release of SP thymocytes from the thymus of newborn CD69 Tg mice by daily injection of antibody (anti-CD69) or CD69 tetramer (data not shown). Thus, blocking the interaction of CD69 with its putative ligand is not sufficient to reverse the effects of CD69 overexpression. These data raise the possibility that CD69 transmits a signal to mature thymocytes that inhibits their export from the thymus.

Cross-linking of CD69 on activated T cells or transfected mature cell lines elicits intracellular signals (e.g. Ca^{2+} influx and Erk activation), suggesting that it can function alone or in concert with the TCR to transduce physiologically relevant signals (33,34). However, we were unable to discern a difference in the Ca^{2+} or Erk activation responses in thymocytes from CD69 Tg and non-Tg mice following CD69 + TCR co-ligation and we did not observe a signaling response in thymocytes from CD69 Tg mice upon CD69 cross-linking (data not shown). In addition, thymocyte selection appeared unaffected in TCR Tg × CD69 Tg mice,

indicating that the TCR signaling response was not influenced by CD69, at least during the period when thymocytes undergo selection in the thymus. Thus, these findings indicate that overexpression of CD69 does not impact signaling pathways downstream of the TCR in thymocytes.

The phenotype of CD69 Tg mice closely resembles that of pertussis toxin (PT) Tg mice (7). In PT Tg mice, thymocyte emigration is blocked, presumably because PT inhibits signaling through G_i-protein-linked chemokine receptors that regulate thymocyte export. As in CD69 Tg mice, functionally mature SP thymocytes accumulate in the thymus of PT Tg mice (7). More recently, it was shown that i.p. injection of PT inhibits the migration of SP thymocytes across the corticomedullary junction into the medulla (6). Interestingly, CD69 associates with a 40-kDa GTP binding (G_α subunit) protein that is inhibited by PT (35). However, unlike mice injected with PT, SP thymocytes are restricted to the medulla in CD69 Tg mice and do not accumulate in the cortex. We also failed to note an obvious migration defect of adoptively transferred SP thymocytes to secondary lymphoid tissues in CD69 Tg mice. Interestingly, whereas SP thymocytes from PT Tg mice fail to migrate from blood into secondary lymphoid organs, SP thymocytes from CD69 Tg mice exhibit an accelerated egress from blood (Fig. 6C). This could reflect an enrichment in mature cells in the SP thymocyte populations from CD69 Tg mice relative to non-Tg mice. Alternatively, CD69 may function to augment chemotaxis initiated by certain chemokine receptors. Since signaling through chemokine receptors can also augment cell adhesion by activating integrins (36,37), this could potentially explain the failure of SP thymocytes to be exported from the thymus.

Notably, no alteration in T cell development has been observed in CD69$^{-/-}$ mice (22). However these results are not necessarily inconsistent with our data. For example, the failure to observe an effect on thymocyte emigration in CD69$^{-/-}$ mice could be explained by the compensatory activity of other molecule(s). In addition, the predicted phenotype of CD69$^{-/-}$ mice would be accelerated export of SP thymocytes, and this phenotype may not be obvious in adult mice since the mechanisms that regulate peripheral T cell numbers and T cell homeostasis may provide feedback that limits thymocyte emigration in T-replete hosts. Detailed analysis of the kinetics of thymocyte maturation and the phenotype of SP thymocytes and lymph node T cells should help to more accurately define the role of CD69 in T cell development.

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Abbreviation

APC	antigen-presenting cell
BrdU	5-bromodeoxyuridine
DN	double negative
DP	double positive
PFA	paraformaldehyde
PT	pertussis toxin
RTE	recent thymic emigrant
SP	single positive
Tg	transgenic

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Phenotypic and functional characteristics of hematopoietic cell lineages in CD69-deficient mice

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AIM/CD69 is the earliest leukocyte activation antigen and is expressed mainly by activated T, B, and natural killer (NK) cells. It is also constitutively expressed by platelets, by bone marrow myeloid precursors, and by small subsets of resident lymphocytes in the secondary lymphoid tissues. The engagement of CD69 by specific antibodies induces intracellular signals, including Ca^{++} flux, cytokine synthesis, and cell proliferation. To investigate the physiological relevance of CD69, we generated mice deficient in CD69 (CD69-/-) by gene targeting in embryonic stem cells. CD69 (-/-) mice showed largely normal hematopoietic cell development and normal T-cell subpopulations in thymus and periphery. Furthermore, studies of negative- and positive-thymocyte selection using a T-cell receptor transgenic model demonstrated that these processes were not altered in CD69 (-/-) mice. In addition, natural killer and cytotoxic T lymphocyte cells from CD69-deficient mice displayed cytotoxic activity similar to that of wild-type mice. Interestingly, B-cell development was affected in the absence of CD69. The $B220^{hi}IgM^{neg}$ bone marrow pre-B cell compartment was augmented in CD69 (-/-) mice. In addition, the absence of CD69 led to a slight increase in immunoglobulin (Ig) G2a and IgM responses to immunization with T-dependent and T-independent antigens. Nevertheless, CD69-deficient lymphocytes had a normal proliferative response to different T-cell and B-cell stimuli. Together, these observations indicate that CD69 plays a role in B-cell development and suggest that the putative stimulatory activity of this molecule on bone marrow-derived cells may be replaced in vivo by other signal transducing receptors. (Blood. 2000;95:2312-2320)

Introduction

The development of the immune response leads to lymphocyte activation by antigens or mitogens, which readily express genes known as early genes (immediate-early genes); these include proto-oncogenes, growth factors, and cytokine receptors.[1] CD69, also termed "Activation Inducer Molecule" (AIM),[2] is expressed on leukocytes during the activation process (reviewed in Sánchez-Madrid[3] and Testi et al[4]). This molecule is a disulfide-linked homodimer (24 kd)[5] that belongs to the type 2 C-type lectin family of surface receptors, characterized by a carbohydrate recognition domain in the C-terminal region.[6,7] The CD69 gene is located in the long arm of mouse chromosome 6, syntenic of chromosome 12 in humans.[6,7] It is found within the region designated "NK complex," which comprises several genes from the family of C-type lectins specific for natural killer (NK) cells.[4] The genomic organization, promoter regions, and transcriptional activity of the human CD69 gene have been reported.[6,8,9] The genetic and biochemical characteristics of mouse CD69 are very similar to its human homologue.[4,7]

Lymphocyte expression of CD69 can be induced in vitro by a wide variety of agents, such as anti-CD3/T-cell receptor (TCR) and anti-CD2 monoclonal antibody (mAb), activators of protein kinase C (PKC), and phytohemagglutinin (PHA). Soon after stimulation of T lymphocytes through the TCR, CD69 messenger RNA levels are transiently increased.[6] CD69 expression is absent in vivo in peripheral blood lymphocytes, but it is expressed by small T- and B-cell subsets in secondary lymphoid tissues, as well as by platelets and bone marrow (BM) myeloid precursors.[10-12] The expression of CD69 by thymocytes undergoing positive selection[13] is associated with the activation process that occurs during thymic development.[14] In this regard, CD69 is a useful marker for defining the molecular map of T-cell development,[15] and a putative role for CD69 in T-cell selection has been postulated by its restricted expression during the late stages of thymocyte development.[13] Because the CD7+ thymic precursors do not express CD69, it appears that its expression is regulated intrathymically. Furthermore, CD69 expression on thymocytes has been associated with apoptosis of these cells.[16] However, T lymphocytes in the inflammatory cell infiltrates of various chronic inflammatory diseases express CD69.[17,18] The expression of CD69 associated with inflammatory processes seems to be induced, at least in part, by proinflammatory cytokines. Up-regulation of CD69 expression is observed in vitro after incubation of lymphocytes with exogenous cytokines.[19,20] Likewise, it has been shown that tumor necrosis factor α is able to promote the transcriptional activity of the 5′ gene region of CD69 gene, thereby inducing CD69-cell surface

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expression.[8] Although the putative ligands for CD69 are so far unknown, it has been demonstrated that CD69 functions as a signal transducer molecule.[2-4] The engagement of CD69 induces an increase in intracellular Ca^{++}, synthesis of cytokines and their receptors, increase in the expression of the proto-oncogenes c-myc and c-fos, as well as cellular proliferation.[2,4,21-23] CD69 thus seems to play an important role in the activation and proliferation of human lymphocytes, although its precise role in leukocyte physiology remains undetermined. Here we have studied this issue by generating CD69-deficient mice. The phenotypic and functional characterization of different hematopoietic cell lineages in the absence of CD69 is the subject of this work.

Materials and methods

Generation of CD69-deficient mice

A genomic DNA clone, containing the complete coding sequence of CD69 and including more than 1.5 kb upstream of translation start site and 9 kb 3′ to exon 5 end, was isolated from a murine 129-Sv phage genomic library (Stratagene, La Jolla, CA). The targeting construct was developed, using the 4.5-kb SalI-EcoRI fragment (long arm) 5′ containing exon 1 (intracytoplasmic domain) and the 3-kb XbaI fragment (short arm) containing the exon 5 untranslated region and 3′ region of genomic 129 v-derived DNA and subcloned in a double-selection vector kindly provided by Drs. D. Milstone and R. Mortensen (Brigham and Women's Hospital, Boston, MA). This vector contains the neomycin-resistance gene (neo) for positive selection of the transfected embryonic stem (ES) cells and a copy of the thymidine kinase gene for the negative selection of randomly integrated constructs. ES cells[24] were grown, transfected, and selected as described.[25] Southern blot was used to identify clones with the expected recombination event, which were analyzed and confirmed using several restriction enzyme digestions (XbaI, BamHI, and EcoRI/EcoRV) and verified using two different probes by Southern blot analysis. The probes used were 5′ flanking, a 2-kb HindIII-EcoRI fragment from the genomic Xba-EcoRI fragment mapping 5′of the targeting construct; 3′Exon-1, a 2-kb HindIII fragment isolated from the genomic fragment SalI-EcoRI carrying the exon 1. Three successfully targeted ES cell clones were aggregated with CD1 morula cells and reimplanted in the uteri of 2.5-day pseudopregnant females. Germline transmission was obtained, and mice were bred to homozygosity on a C57BL/6 genetic background. Consecutive litters were analyzed by polymerase chain reaction using peripheral blood samples.

Mice

Mice were bred at the Centro Nacional de Biotecnologia (Madrid, Spain) under specific pathogen-free conditions. Mice used for experiments were 8 to 16 weeks of age, and all experiments used either littermate controls or age-matched litters whose parents were littermates. In some cases, mice bearing the TCR transgene F5[26] were intercrossed with CD69 (-/-) mice to obtain F5 transgenic TCR, CD69 (-/+) heterozygous mice that, when intercrossed, produced F5 TCR, CD69 (-/-) and F5 TCR, CD69 (+/+) littermates.

Peptide treatment of F5 TCR transgenic mice

Mice were injected intraperitoneally with the peptide 9-mer Ala-Asn-Glu-Asn-Met-Asp-Ala-Met NP-[366-374] from the influenza virus nucleoprotein A/NT/60/68 in phosphate-buffered saline (PBS) at the indicated concentration.

Cell culture

All cell cultures were maintained in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, 10 mmol/L HEPES, 10^{-5} mol/L 2-mercaptoethanol, 100 U/mL penicillin/streptomycin, 2 mmol/L L-glutamine, and 1 mmol/L sodium pyruvate at 37°C, 5% CO_2.

Determination of hematological parameters

To measure blood cell parameters, whole blood was collected in tubes containing K-EDTA and analyzed on an automatic hematology counter (Technicon H*1E, Bayer, Tarrytown, NY).

Flow cytometry analysis

BM, thymus, spleen, lymph node, peripheral blood, and peritoneal exudate cell suspensions were obtained from CD69 (-/-) or control C57BL/6 littermates. Cells were stained with FITC-, PE-, TC-, or SPRD-conjugated antibody reagents or indirectly with biotinylated antibodies followed by streptavidin-FITC, -PE, -TC, or -SPRD. The antibodies used for staining were anti-CD69 (H1.2F3), anti-CD2 (RM2-5), anti-CD3 (17A2), anti-CD4 (GK1.5), anti-CD5 (53-7.3), anti-CD8 (53.6.7), anti-CD25 (PC61), anti-CD44 (IM7), anti-NK (D × 5), anti-TCRαβ (H57-597), anti-Vβ3 (53), anti-Vβ8 (F2.3.1), anti-Vβ11 (RR3-15), anti-Vα2 (B20.1), anti-TCRγδ (GL3), anti-B220/CD45R (RA3-6B2), anti-CD43 (S7), anti-BP1 (6C3), anti-immunoglobulin (Ig) M (AF6-78), anti-IgD (AMS15.1.5), and anti-HSA (M1/69) (PharMingen, San Diego, CA). Propidium iodide was used at 5 μg/mL to detect dead cells. Cells were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Cell staining and flow cytometry were performed according to standard methods, and, for 4-color analysis, a total of 50 000 events were acquired.

Analysis of hematopoietic progenitors

Progenitors of the granulocyte/macrophage lineage were assayed by culturing 10^5 BM cells in Bactoagar medium (Difco, Detroit, MI), as previously described.[27] Briefly, cells were resuspended in Iscove's modified Dulbecco's medium (Gibco-life Sciences, Gaithersburg, MD) supplemented with 25% horse serum (Gibco) and 10% WEHI-3b conditioned medium as an interleukin-3 (IL-3) source, and then mixed with Bactoagar (0.3% final concentration) and seeded into 35-mm plastic tissue culture dishes (Nunc, Roskilde, Denmark). The megakaryocyte colony-forming units were assayed in serum-free cultures. BM cells (3×10^5 cells per dish) were cultured on Iscove's modified Dulbecco's medium supplemented with 10 mg/mL bovine serum albumin, 3 μg/mL transferrin, 25 μg/mL soy bean lipids (all from Boehringer Mannheim, Mannheim, Germany), 45 μg/mL linoleic acid (Sigma, St Louis, MO), 7.8 μg/mL cholesterol (Sigma), 110 μg/mL sodium pyruvate (Sigma), 10^{-4} mol/L L-thioglycerol (Fluka, Buchs, Switzerland), 2×10^{-2} mmol/L glutamine (Gibco), and 20% WEHI-3b conditioned medium. The pre-B colony-forming units were assayed by culturing 10^5 BM cells in methylcellulose culture medium supplemented with recombinant human IL-7 (Methocult M3630; StemCell Technologies, Vancouver, Canada) as described.[28] In all instances, triplicates of each sample were seeded and incubated for 7 days at 37°C in 95% humidified atmosphere with 5% CO_2 in air. Colonies were scored under a dissecting microscope, and, in the case of the megakaryocyte cultures, colonies were stained with acetylcholinesterase prior to scoring.[29] The exogenous spleen colony forming-units were assayed as described previously.[30] Briefly, groups of 10 C57Bl/6 mice were irradiated with a split dose of 9 Gy (2 doses of 4.5 Gy spaced 4 hours apart), using Philips MG 324 x-ray equipment (Philips, Hamburg, Germany) at 300 kV, 10 mA, and a dose rate of 1.03 Gy/min. Each recipient was injected with 5×10^4 cells through the lateral vein tail. Twelve days after transplantation, recipients were killed and their spleens removed and fixed in Telleyeniczky's solution. Spleen colonies were then determined using a dissecting microscope.

Cell proliferation assays

Cell suspensions were prepared from spleen, lymph node, or thymus and cultured in triplicate in the presence of plate-bound anti-CD3 antibody, staphylococcus enterotoxin B, CD40L, anti-IgM, or lipopolysaccharide (LPS) at various concentrations in culture medium for 3 days. Cells were pulsed with [^{3}H]dT (1 μCi/well) 12 hours before harvesting onto glass fiber filters for determination of [^{3}H]dT. For measurement of lymphocyte proliferation in vivo, mice were intraperitoneally injected with 0.6 mg bromodeoxyuridine (BrdU) in 100 μL PBS 18 hours before flow cytometry analysis of BrdU incorporation.[31]

^{51}Cr release assay

Specific NK cell and cytotoxic T lymphocyte (CTL) activity was determined using a standard ^{51}Cr release assay. Splenocyte suspensions were prepared and erythrocytes were lysed by NH_4Cl treatment. Cr-labeled (100 μCi sodium chromate) YAC-1, NK-resistant (RMA), or NK-sensitive

(RMA-S) target cells were plated with the appropriate effector cells at different ratios. NK cytotoxic activity was measured at 2.5 hours. In CTL experiments, splenocytes stimulated in vivo for 3-4 days by intraperitoneal injection of F5 peptide (18 nmol) in PBS were used as effector cells. RMA and RMA-S unsensitized and F5 peptide-sensitized by preincubation with 100 nmol NP 366-374 in 1 mL medium overnight at 26°C were used as targets cells. Cytotoxicity was measured after an incubation period of 4 hours. The percentage of specific lysis was calculated as (sample cpm − spontaneous cpm)/(maximal cpm − spontaneous cpm)] × 100.

Immunizations and enzyme-linked immunosorbant assay (ELISA)

Age- and sex-matched mice were immunized intraperitoneally with 10 μg of 2,4-dinitrophenyl (DNP)-keyhole limpet hemocyanin (DNP-KLH) in complete Freund's adjuvant (CFA) or in alum, or intravenously with DNP-KLH in PBS at day 0. Each mouse was boosted 7 days later using the same protocol. Blood was collected at days 6 and 14 after the first immunization. Other mice were immunized intraperitoneally with 10 μg LPS-TNP (Sigma) in PBS at day 0, and bled at day 0 and 14. NP-specific antibodies were measured by ELISA using DNP-ovalbumin-coated plates at 3 μg/mL. Previously tested horseradish peroxidase-labeled isotype-specific anti-mouse immunoglobulin antibodies (Southern Biotechnology Associates, Birmingham, AL) were used in these assays. Sera were diluted 1:200 and 1:2000 and immunoglobulin levels were analyzed.

Immunohistochemistry

Frozen spleen, lymph node, and Peyer's patch sections from DNP-KLH immunized CD69-deficient and wild-type mice (day 10 after immunization) were stained with the indicated biotinylated antibodies, followed by streptavidin-peroxidase. Sections were developed with diamino-benzidine and counterstained with Mayer's hematoxylin.

Results

Generation and characterization of lymphoid populations in CD69 (-/-) mice

The CD69-mouse genomic DNA consists of 5 exons and 4 introns and is approximately 9 kb in length.[7] To disrupt CD69-gene expression by homologous recombination in ES cells, a targeting vector was constructed by replacing with the neomycin-resistance gene, a fragment of genomic DNA of 4 kb containing exons 2, 3, 4 and translated region of the exon 5 (Figure 1A). Two chimeric mice were obtained by morula aggregation, one of which successfully contributed to the germ line. Homozygous mice were obtained by interbreeding of heterozygous mice (Figure 1B). CD69 (-/-) mice appeared normal, with no apparent developmental defects. Mice were fertile and no obvious defects were appreciated in any organ analyzed after autopsy.

To demonstrate the lack of CD69 expression in CD69 (-/-) mice, thymocytes were stained with anti-CD69 mAb and CD2 as control and analyzed by flow cytometry. As expected, CD69 expression was absent in CD69 (-/-), while in wild-type CD69 (+/+) mice, approximately 29% of $CD2^+$ thymocytes coexpressed CD69 (Figure 1C). Intermediate CD69 expression was found in heterozygous CD69 (+/−) mice (Figure 1C). Moreover, spleen, lymph

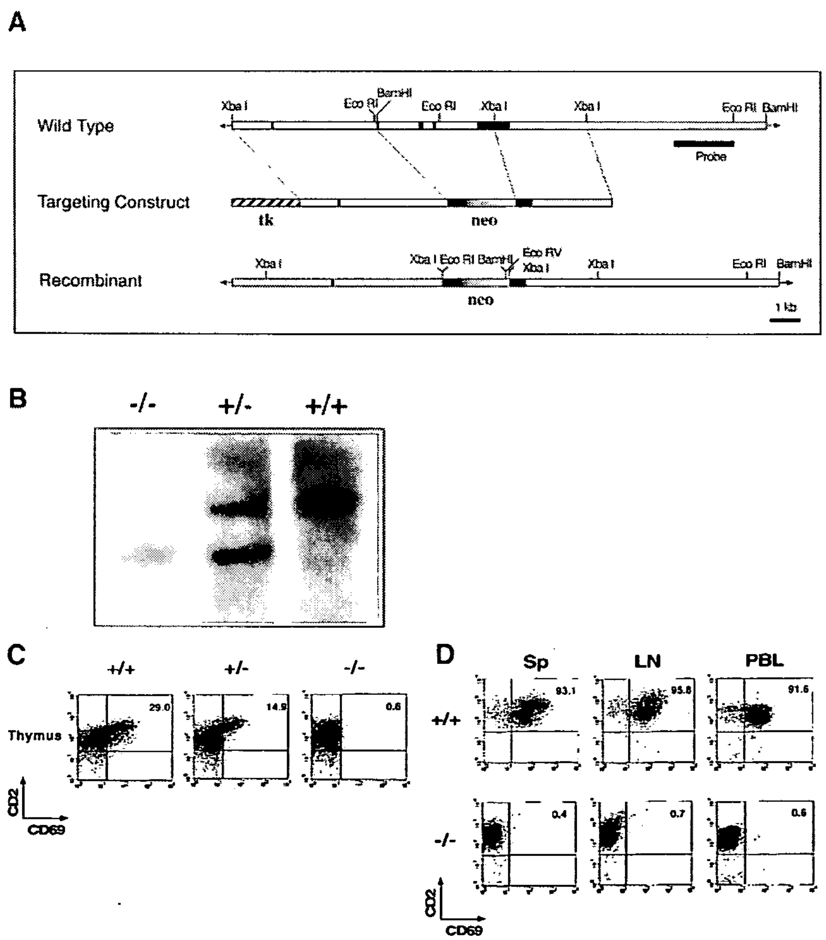


Figure 1. CD69 gene disruption by homologous recombination. (A) Schematic representation of the partial restriction maps of the mouse genomic CD69 locus, the targeting construct, and the targeted allele. The exons of the wild-type CD69 gene are shown as solid bars, boundaries of homology between the wild-type gene and the targeting construct are denoted by thin lines. (B) Southern blot analysis of BamHI-digested tail DNA from wild-type (+/+) and heterozygous (+/−) or homozygous (-/-) CD69 mice were analyzed with a 2-kb HindIII-EcoRI fragment from the genomic Xba-EcoRI fragment mapping 5′of the targeting construct. The unmutated CD69 gene produced a 12-kb fragment, whereas the mutated allele results in a 9-kb fragment. (C) CD69 expression in wild-type, CD69 (+/−), and in CD69 (-/-) mice. Flow cytometry analysis of CD69 expression in thymus from wild type (+/+), heterozygous(+/−), or homozygous (-/-) CD69 mutant mice. (D) CD69 expression in spleen, lymph node, and peripheral blood lymphocytes from wild type (+/+) or CD69 (-/-) mutant mice. Cells were activated with PMA at 10 ng/mL for 15 hours. Cells were double-stained with anti-CD69 and anti-CD2 labeled monoclonal antibodies and analyzed by flow cytometry.

node, and peripheral blood T cells from CD69 (-/-) mice activated with PMA did not express CD69 (Figure 1D). All these results confirmed the inactivation of the CD69 gene and the inheritance of this trait at the expected Mendelian ratio.

Comparison of CD69 (-/-) and CD69 (+/+) mice from the same litter showed no significant differences in the $CD3^+$ lymphoid populations in thymus, spleen, and lymph nodes (Figure 2A, and data not shown). Moreover, no differences were appreciated in the major T-cell subsets, as assessed by CD4 and CD8 expression (Figure 2B). Similar results were obtained using other cell markers such as CD2, CD5, CD25, and CD44 (data not shown). Mature and immature thymic subsets in CD69 (-/-) mice showed a normal pattern of development (Figure 2B). Proportions of mature TCRαβ and TCRγδ were similar in CD69 (-/-) and CD69 (+/+) mice (data not shown). Likewise, normal cell numbers and a normal T:B lymphocyte ratio were observed in the peripheral lymphoid organs of CD69 (-/-) deficient mice (Figure 2B). Lymphoid organs, including thymus, spleen, lymph nodes, and Peyer's patches from CD69 (-/-) mice, had a normal appearance by histological analysis (Figure 2C, and data not shown). No significant differences in other cell subsets from peripheral lymphoid organs were found, as determined with a panel of mAb specific for B cells (B220, IgM, IgG, CD43), granulocytes (Gr), and monocytes (CD11b, CD71, I-Ab, ICAM-I). Peritoneal exudate cells were also present in normal proportions in CD69 (-/-) mice (data not shown).

Hematopoiesis in CD69 (-/-) mice: altered pre-B immature cells

Analysis of CD69 expression in normal BM revealed its presence in the $B220^+$ pre-B cells (Figure 3A). Interestingly, CD69 was expressed with high intensity on 20%-30% of $B220^+$ pre-B cells; it was, therefore, of interest to analyze B cells at different maturation stages within CD69 (-/-) mouse BM. Increased percentages of pre-B ($B220^{+int}$, IgM^{neg}) and immature B ($B220^{+int}$, IgM^+) cells were consistently detected in CD69 (-/-) mice compared with wild-type mice. The pre-B cell compartment, defined as $B220^{+int}$ IgM^{neg}, was the most affected, whereas $B220^{+int}$ IgM^+ pre-B and immature B cells were only moderately augmented (Figure 3B). The overall increase in the $B220^{+int}$ IgM^{neg} lymphoid cell subset was statistically significant. The relative change mean for this subset was 1.523 ($P < .01$; Student t test, sample size = 20). The detection of another pre-B cell marker (the Heat Stable Antigen, HSA) confirmed the difference in this B-cell subset between wild-type and CD69-deficient mice (Figure 3C). These differences correlate with a slight lymphocytosis in CD69 (-/-) mice (1.35 relative change mean, $P < .01$). In contrast, the mature B-cell compartment ($B220^{bright}$ IgM^+) was not significantly altered. Analysis of pro-B cells defined by CD43, BP-1, and HSA markers revealed no alteration in CD69 (-/-) mice (data not shown). Our results, therefore, show that CD69 is constitutively expressed on B-cell precursors and appears to play a role in cell maturation. To further analyze the effect of the disruption of CD69 gene in B-cell development, clonogenic assays with BM cells from CD69 (-/-) and wild-type mice were performed, but no significant differences were found (Table 1).

The expression of CD69 has been described on BM myeloid precursors.[10] In CD69 (-/-) mice, erythroid and myeloid precursors were normal, according to both their number and specific surface markers (Table 1, and data not shown). Clonogenic assays with BM cells of myeloid progenitors showed no significant differences in the values of granulocyte/macrophage and megakaryocyte colony-forming units obtained between CD69 (-/-) and wild-type mice. Similarly, no differences were found in analyses of a more primitive and multipotential precursor by exogenous spleen colony-forming unit assays (Table 1). Hematologic analyses demonstrated that the cellular components of peripheral blood were normal in number and distribution (data not shown). In addition, although CD69 is expressed in a constitutive manner in normal platelets, platelet numbers were similar in the CD69 (-/-) and wild-type mice.

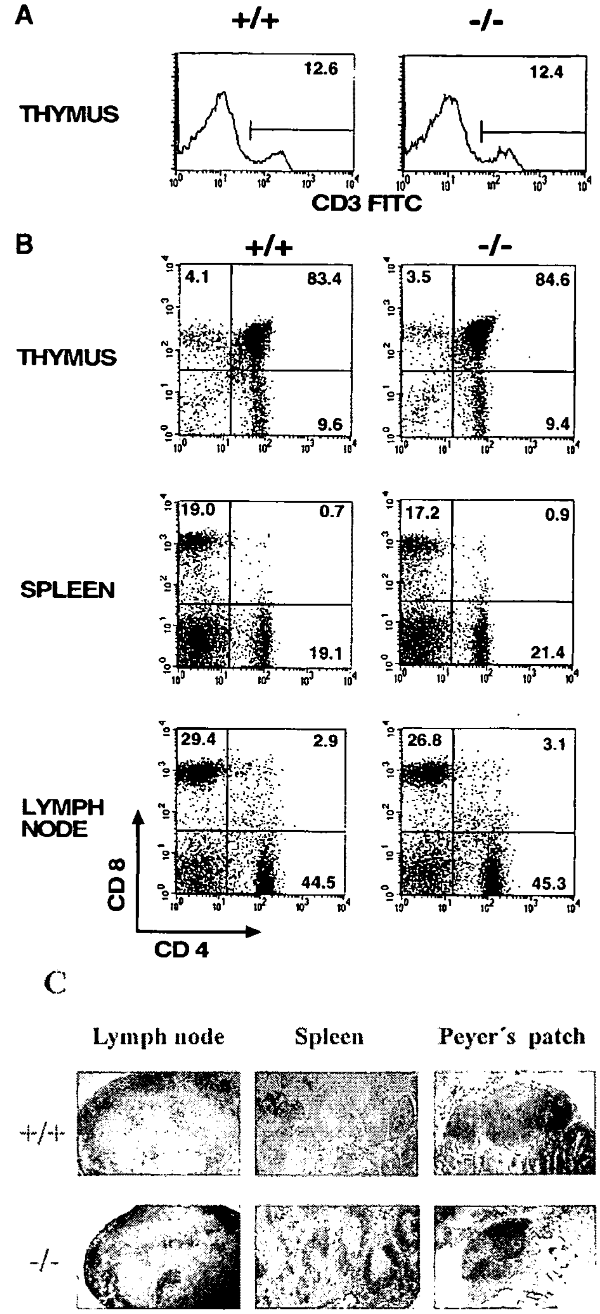


Figure 2. Lymphocyte subpopulation analysis in CD69 (-/-) and wild-type mice. Cells from lymphoid organs were obtained from CD69 (-/-) mice and wild-type littermate controls, then immunofluorescently stained and analyzed using flow cytometry. (A, B) CD3 expression on thymocytes and CD4 and CD8 markers on thymocytes, splenocytes, and lymph node cells. Numbers indicate the percentage of lymphoid populations. Data from a single mouse are shown, they are representative of more than five mice per group and from more than one litter. (C) Immunochemical analysis of lymphoid organs from CD69 (-/-) mice. Frozen sections of lymph nodes and spleens from unimmunized mice were immunostained for immunoglobulin M. Peyer's patches obtained from mice 10 days after challenge by intraperitoneal injection of 10 μg of alum absorbed 2,4-dinitrophenyl-keyhole limpet hemocyanin mice were stained with anti-CD5.

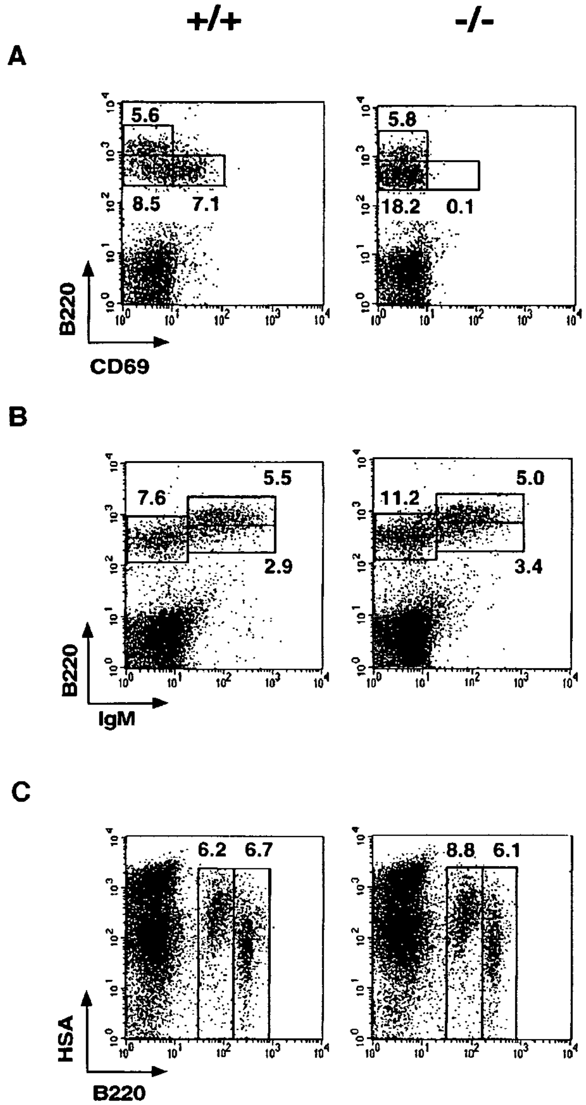


Figure 3. Phenotypic analysis of B-cell precursors in CD69 (-/-) and wild-type bone marrow. (A-C) Two-color immunofluorescence analysis with anti-B220 and anti-CD69 monoclonal antibody shows that $B220^+CD69^+$ cells are enriched in the $B220^{int}$ lymphocyte fraction. In CD69 (-/-) mice, the increase in IgM^-B220^+ and HSA^+B220^+ cell subsets is also shown.

Finally, analyses of different serum biochemical parameters in CD69 (-/-) mice showed no abnormality in the protein, lipid, and enzyme content studied (data not shown).

Positive and negative selection of thymocytes

The definition of thymocyte subsets is currently based on the detection of different molecules (TCR, CD4, CD8), including CD69. CD69 is absent in double-positive $TCR^{neg/lo}$ cells and is not expressed in TCRα-deficient mice and in major histocompatibility complex Class I and Class II double-deficient mice.[32] $CD4^{lo}CD8^{lo}$ and $CD4^{lo}CD8+$ TCR^{int} cells express CD69. Therefore, CD69 expression is induced just after the initiation of TCRαβ positive selection in thymocytes, when these cells are signaled via their TCR.[13,33] To study the role of CD69 in thymocyte selection, CD69 (-/-) TCR F5 transgenic mice were generated. The F5 receptor is specific for influenza virus A/NT/60/68 nucleoprotein NP $^{366\text{-}379}$ and utilizes the Vα4 and Vβ11 TCR gene segments. CD8+T cells expressing F5 are positively selected in TCR transgenic H-$2D^b$ wild-type mice. The process of positive selection into the CD8 compartment was not altered in F5 TCR CD69 (-/-) mice (Figure 4A). Both F5 TCR CD69 (-/-) and F5 TCR wild-type mice thus have a high CD8:CD4 cell ratio. Accordingly, the thymocyte expression of transgenic Vβ11 chain by T cells was high in both mice (not shown).

Negative selection was induced in F5-TCR transgenic mice by intraperitoneal injection of the antigenic peptide of the influenza nucleoprotein (NP) 366-374.[26] This negative selection was reflected in a threefold reduction of thymus cellularity, a decrease in double-positive thymocytes, a depletion of Vβ11-bearing T cells, and an apparent increase in $CD4^-CD8^-$ thymocytes detected at 2 or 4 days after peptide administration. A similar pattern of expression of CD4, CD8, Vβ11, and CD25 was observed in both F5 TCR CD69 (-/-) and F5 TCR CD69 (+/+) mice (Figure 4A, and data not shown). Positive and negative selection of thymocytes were, therefore, unaltered by CD69 deficiency.

Lymphocyte proliferative responses

To explore the possible involvement of CD69 in B- and T-cell functions, we first determined the proliferative response of thymus, spleen, and lymph node cells from CD69 (-/-) mice to different stimuli. Cells from CD69-deficient mice displayed proliferative responses to T-cell-specific stimuli, such as anti-CD3, Con-A, and staphylococcus enterotoxin B, similar to those of cells from wild-type mice (Table 2). In addition, lymph node and spleen cells from CD69 (-/-) mice showed a normal response to B-cell stimuli, such as LPS and CD40L+ IL-4 (Table 2 and data not shown). These results indicated that CD69 is not necessary for the proliferation of lymphocytes induced by different stimuli.

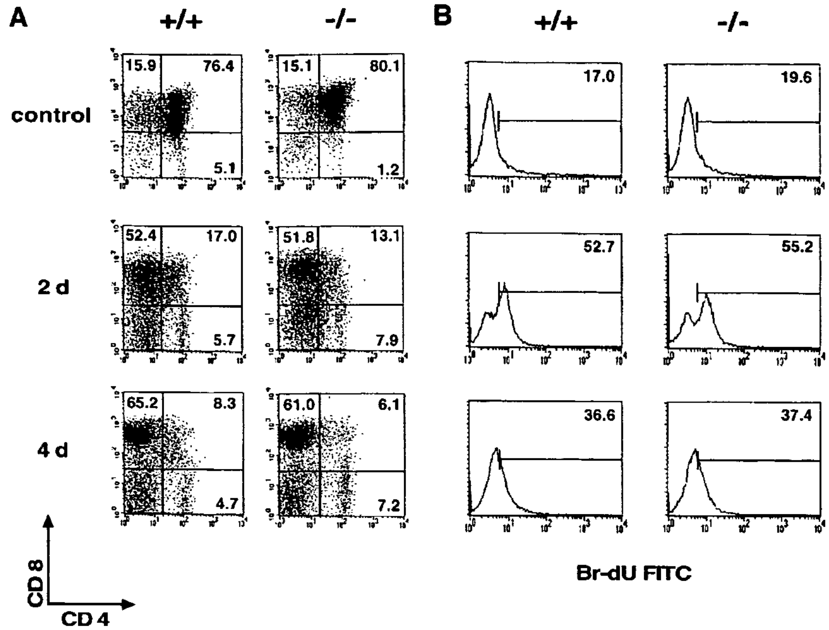
To assess the in vivo role of CD69 in T-cell proliferation, lymph node and spleen transgenic CD8+ T-cell expansion was studied in TCR F5 transgenic CD69 (-/-) mice by BrdU incorporation. It has been described that exposure of mice transgenic for a F5 TCR to $NP^{366\text{-}374}$ peptide results in expansion and activation of peripheral Vβ11-bearing CD8 T cells.[26] Both F5 TCR CD69 (-/-) and F5 TCR wild-type mice showed a comparable high rate of peripheral CD8 T-cell proliferation (Figure 4B, and data not shown). These results showed that T cells are able to proliferate in vivo in response to a specific antigen in the absence of CD69. T-cell cytokine production was analyzed by intracytoplasmic staining of PMA-activated Th1-derived spleen cells. A comparable cytokine synthesis profile was observed in cells from CD69 (-/-) and wild-type mice (data not shown).

Table 1. Comparative analysis of the hematopoietic progenitors present in femoral bone marrow of CD69 (−/−) and wild-type mice

	Cells per Femoral BM (×10^6)	CFU-GM	CFU-Meg	CFU-Pre-B	CFU-S_{12}
Wild-type mice	15.56 ± 1.00	108 ± 6	2.50 ± 0.47	29.00 ± 5.95	13.00 ± 1.50
CD69 (−/−) mice	16.88 ± 1.51	108 ± 5	3.50 ± 0.76	20.04 ± 4.26	15.00 ± 1.10

Number of colonies per 10^5 bone marrow (BM) cells. Samples from a total of 14 mice per group were individually analyzed. The mean ± standard error corresponding to each group is shown. CFU-GM = granulocyte/macrophage colon-forming units; CFU-Meg = megakaryocyte colony-forming units; CFU-Pre-B = pre-B colony-forming units; CFU-S_{12} = exogenous spleen colony forming-units.

Figure 4. Analysis of thymic selection and in vivo proliferation of mature T cells in CD69 (-/-) F5 T-cell receptor (TCR) transgenic mice. (A) Two-color flow cytometry analysis was performed in thymocytes of F5 TCR transgenic mice untreated or after the administration of NP366-374 as described in Materials and methods section. (B) The proliferative capability of mature T cells in CD69 (-/-) mice was analyzed in vivo after bromodeoxyuridine (BrdU) treatment as in the Materials and methods section. Numbers indicate the percentage of positive, BrdU-incorporating lymph node cells. Data shown are representative of results obtained in three mice per group and from more than one litter.



Cytolytic activity in CD69 (-/-) mice

Previous studies[33] suggested that CD69 may be involved in the regulation of cytolytic activity in NK and Tγδ lymphoid cells. We, therefore, analyzed the cytolytic activity of CD69 (-/-) lymphocytes against NK-sensitive and NK-resistant target cells. The number and phenotype of splenic NK and $CD8^+$ cytotoxic cells were found to be similar in both CD69 (-/-) and wild-type mice (data not shown). We assayed two different effector cells, spleen cells cultured in the presence of phytohemagglutinin plus IL-2, and unstimulated spleen cells. CD69 (+/+) and CD69 (-/-) splenocytes displayed similar cytolytic activity against RMA-S or RMA syngeneic target cells (Figure 5A).

Because a major CD8 T-cell subset differentiates in F5 TCR transgenic lymphocytes on the C57BL/6 ($H\text{-}2^b$ haplotype) genetic background, we compared the lytic activity of in vivo activated CTL from CD69 (-/-) and CD69 (+/+) F5 TCR mice against RMA-S and RMA cells loaded with NP peptide. We found no differences in peptide-specific lysis mediated by splenocytes from either type of mice when the lytic assay was performed using an optimal concentration peptide loading (100 μmol/L) (Figure 5B).

Table 2. In vitro proliferative response

Stimuli	Thymus	Spleen	Lymph Node
—			
WT	244 ± 20	16 062 ± 1117	2483 ± 196
CD69 (−/−)	141 ± 20	17 566 ± 892	1886 ± 349
ConA			
WT	6635 ± 714	51 101 ± 1684	27 170 ± 2532
CD69 (−/−)	6181 ± 920	48 351 ± 699	25 802 ± 2532
α-CD3			
WT	5252 ± 1702	117 254 ± 15 912	71 121 ± 16 090
CD69 (−/−)	5516 ± 497	95 288 ± 19 426	54 115 ± 7342
SEB			
WT	1007 ± 237	92 218 ± 4051	65 274 ± 5013
CD69 (−/−)	692 ± 84	110 317 ± 27 564	40 262 ± 5636
LPS			
WT	3196 ± 116	61 186 ± 3783	118 482 ± 5326
CD69 (−/−)	1631 ± 203	70 372 ± 5650	79 867 ± 10 036

Mononuclear cells obtained from thymus, spleen, and lymph nodes from CD69 (−/−) mice and control littermates were stimulated in a proliferation assay. The results shown are the mean of triplicate cultures and are representative of six separate experiments. LPS = lipopolysaccharide; SEB = staphylococcus enterotoxin B; WT = wild type.

Antibody responses

The possible role of CD69 in adaptive B-cell responses was analyzed. Different immunization protocols were used to evaluate the humoral immune response of CD69-deficient mice against thymus-dependent (TD) and -independent antigens. For TD B-cell responses, age- and sex-matched CD69 (+/+) and CD69 (-/-) mice of the same progeny were divided into groups receiving 10 μg of DNP-KLH in PBS intravenously, or adsorbed to alum or mixed with CFA intraperitoneally. Basal immunoglobulin concentrations were in the same range in all mice tested prior to immunization (Figure 6). CD69-deficient mice were able to mount DNP-specific primary and

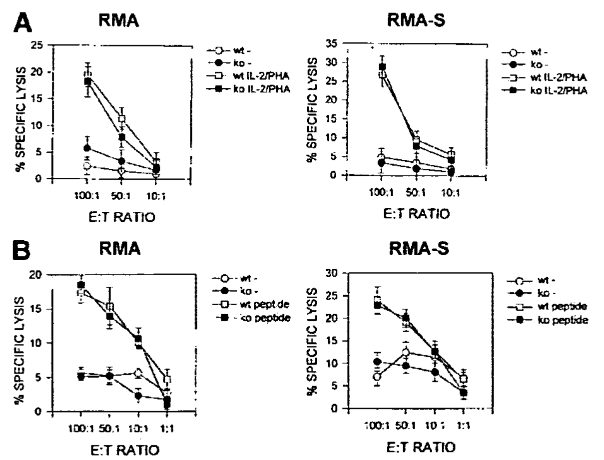


Figure 5. Cytotoxic T lymphocyte activity in CD69 (-/-) mice. (A) Natural killer (NK) cytotoxity against NK-resistant and NK-sensitive cells using unfractionated spleen cells. Cytotoxic activity was assayed in the presence or absence of phytohemagglutinin (PHA) plus IL-2. Specific lysis is shown for three mice per group, representative of five experiments. (B) Nucleoprotein (NP) peptide-specific cytotoxic response of spleen cells from F5 CD69 (+/+) and F5 CD69 (-/-) mice, treated with influenza virus nucleoprotein peptide as in Materials and methods section. Cells were assayed in the presence or absence of the NP366-374. Results shown are the average of three mice per group, representative of three experiments.

secondary responses of all immunoglobulin isotypes. The magnitude of IgG1, IgG2b, and IgG3 antibody responses was similar in the wild-type and CD69 (-/-) mice. A slight increase in the IgG2a primary response was observed in CD69 (-/-) mice, regardless of the immunization protocol used (Figure 6A). IgG2a anti-DNP antibodies were also slightly increased in secondary responses when immunization was intravenous. Moreover, a slightly enhanced IgM response was observed in immunized CD69 (-/-) mice, but the differences in antibody titers were not statistically significant between CD69 (-/-) and CD69 (+/+) mice. Nevertheless, CD69-deficient mice produced specific antibodies to the DNP-LPS thymus-independent antigen at a similar level to that of wild-type mice (Figure 6B). As was found for TD antigens, immunized CD69 (-/-) mice produced slightly more anti-DNP-LPS IgM and IgG2a than wild-type mice.

It has been postulated that CD69 plays a role in T-B cell collaboration, as it is highly expressed by germinal center CD4 T cells[12]; we, therefore, performed immunohistochemical stainings of spleen and lymph node sections after DNP-KLH immunization. We found that mAb specific for IgM, B220, CD3, and CD5 exhibited a similar staining pattern in lymph node sections from CD69 (-/-) and CD69 (+/+) mice. T- and B-cell areas and germinal centers were clearly appreciated in immunized lymph nodes from both types of mice (Figure 2C, and data not shown). In addition, no differences were observed in T cells located in the periphery of lymphoid follicles and B cells within the follicles. Likewise, no significant differences were detected in spleen sections, Peyer's patches, and intraepithelial lymphocytes from CD69 (-/-) and CD69 (+/+) immunized mice (Figure 2C, and data not shown).

Discussion

The pattern of expression of CD69 as well as its putative role as a signal transducing receptor on leukocytes points to an important role for CD69 in the biology of these cells. In addition, CD69 expression during thymocyte and B-cell development, as well as during activation of mature lymphocytes, strongly suggests its involvement in the differentiation and proliferation of these cells.[3,4] We explored the physiological role of CD69 in vivo and studied the phenotypic and functional characteristics of lymphocyte development in CD69 (-/-) mice. T-lymphocyte ontogeny was apparently normal, whereas a subtle alteration in B-cell development was detected. CD69 is constitutively expressed by a significant percentage of B-cell precursors in normal BM, and the pre-B-cell subset was significantly augmented in CD69-deficient mice. Furthermore, CD69 (-/-) mice exhibited a slight but significant increase in the humoral immune response against a TD antigen. No additional alterations were found in any other cell compartments or functions studied, including NK and T cells, macrophages, granulocytes, and platelets.

It has been described that CD69 is detected on activated B cells.[12] The CD69 expression by BM B-cell precursors at the $B220^+$ pre-B-cell stage found by us is thus probably a consequence of the cell activation process that occurs during B-lymphocyte development. CD69 could be involved in the positive selection of B cells in BM, paralleling what it is thought to occur during T-cell development.[32,34] It has been described that surface immunoglobulin crosslinking on mature B cells induces CD69 expression. It is feasible that in pre-B cells, which do not bear surface immunoglobulins, different activatory stimuli such as cytokines are able to induce CD69 expression. In addition, the possibility of CD69 expression induction on pre-B cells by the interaction of a pre-B-cell receptor with its putative ligand cannot be ruled out. Interestingly, CD69 (-/-) mice showed a significant increase in the number of pre-B and immature B cells compared with CD69 (+/+) wild-type mice. These data support a regulatory role for CD69 in B-cell differentiation. It is feasible that CD69 acts as a signal transducing molecule in pre-B cells, with a role in the induction of differentiation of these cells. Under such circumstances, the absence of CD69 may be responsible for slow pre-B-lymphocyte differentiation, with accumulation (and increase in the number) of cells at this differentiation stage. An alternative, but not exclusive, possibility is that CD69 is involved in the induction of apoptosis associated with pre-B-cell activation (activation-induced cell death). In this case, the absence of CD69 would be responsible for the augmented number of pre-B cells in CD69 (-/-) mice due to defective deletion of these cells. This possibility is supported by a report on the association between CD69 expression and programmed cell death in thymocytes.[16] Another possibility is that

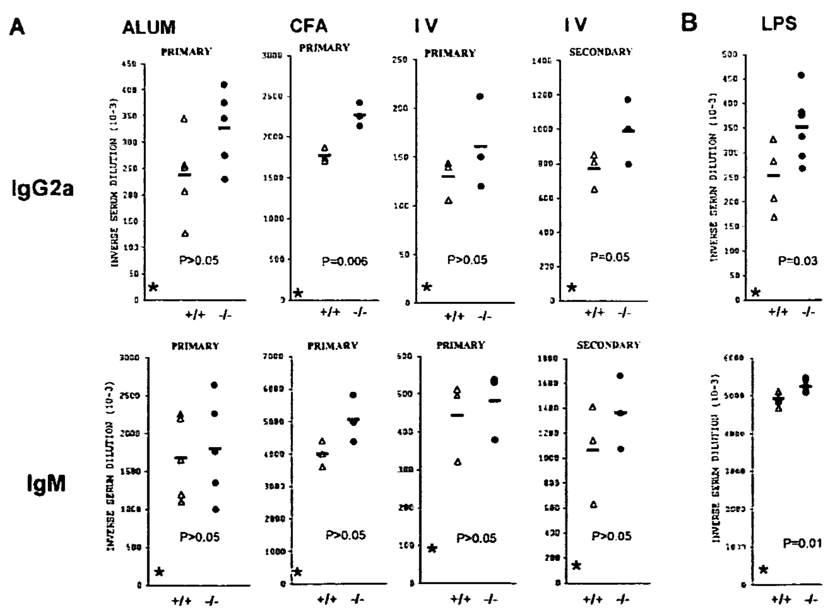


Figure 6. Specific antibody responses in CD69 (-/-) mice. Several immunization protocols were used to assess the immunoglobulin responses in CD69-deficient mice. The responses of individual mutant (filled circles) and wild-type (open triangles) mice are shown. (A) T-cell-dependent B-cell response. Results shown are from different groups: (IV), immunized with 2,4-dinitrophenyl (DNP)-keyhole limpet hemocyanin (DNP-KLH) in phosphate-buffered saline (PBS), (ALUM) immunized intraperitoneally with DNP-KLH adsorbed to alum, and complete Freund's adjuvant (CFA) immunized intraperitoneally with DNP-KLH mixed with CFA. CD69 (-/-) mice immunized with DNP-KLH mixed with CFA and DNP-KLH in phosphate-buffered saline produce augmented amounts of immunoglobulin (Ig) G2a ($P \leq 0.05$, two-way analysis of variance). (B) T-cell-independent B-cell response. DNP-LPS CD69 (-/-) mice produce increased amounts of IgM (top panel) and IgG2a (bottom panel) antibodies after immunization. IgM and IgG2a anti-DNP titers differed significantly ($P < .05$) between the two groups (+/+ and -/-) by two-way analysis of variance. Other DNP-specific antibody isotypes were at similar levels in CD69 (-/-) and wild-type mice (not shown).

CD69 might act in pre-B cells as an inhibitory membrane receptor, generating negative regulatory signals that would constrain cell proliferation. Although all available information on CD69 strongly suggests that this molecule acts as a costimulatory receptor with an important role in lymphocyte activation and proliferation (reviewed in Sánchez-Madrid[3] and Testi et al[4]), we think it is possible that CD69 may exert an inhibitory effect in some cells. In this regard, the CD94-NKG2 lectin-like receptors, which exhibit some degree of homology with CD69, are inhibitory/triggering molecules in NK cells.[35] CD94-NKG2A is thus an inhibitory receptor coupled to SHP tyrosine phosphatases, whereas CD94-NKG2C forms a triggering complex in these cells. Nevertheless, other membrane receptors, such as those for IL-10 and transforming growth factor β, exert stimulatory and inhibitory effects on different leukocyte subsets.[36-39]

Other inhibitory/stimulatory coreceptors have been described on lymphoid cells, such as the killer inhibitory receptors that are expressed, aside from NK cells, by some T lymphocytes, in which they regulate TCR-dependent functions.[40] Finally, the CD81 tetraspanin regulates lymphocyte functions, acting as positive or negative mediator of lymphocyte proliferation depending on the type of stimulus and modulating the immunoglobulin isotype balance.[41] It is thus feasible that CD69 exerts an inhibitory effect on certain cell types at specific differentiation stages. In addition, the absence of CD69 could lower the signal threshold necessary for the effect of receptors involved in negative signaling on B-cell activation/proliferation such as CD22 and FcγRII.[42,43] It is, therefore, conceivable that CD69 acts in pre-B cells by modulating the signaling function of other membrane receptors involved in cell activation. In this regard, CD22 is described as both a positive and negative modulator of B-cell antigen receptor complex signal transduction in mature and immature B cells.[42]

The lack of effect of CD69 deficiency on T-cell function is of interest. CD69 has been also termed "Activation Inducer Molecule" and is expressed on leukocytes during and after their activation.[2-4] CD69 expression can be induced in vitro on lymphocytes by several stimuli, including anti-CD3 and anti-CD2 mAb, PMA, and phytohemagglutinin. The functional characteristics and molecular structure of CD69, a type 2 C-type lectin with a carbohydrate recognition domain in the C-terminal region,[6,7] as well as its expression pattern, suggest its involvement in cell activation. Different data support the role of this molecule as a signal transducing receptor in leukocytes. Although the putative ligands for CD69 have not been characterized yet, it has thus been found that the engagement of CD69 with mAbs induces an increase in intracellular Ca^{++}, as well as cytokine synthesis and expression of proto-oncogenes. When the effect of CD69 engagement is combined with a PKC activator, lymphocyte activation proceeds to DNA synthesis and cell proliferation is observed.[2,22] We, therefore, expected to detect abnormalities in T-cell activation or development in CD69-deficient mice. Despite CD69 expressed during thymocyte differentiation in normal mice, no significant changes were observed in T-lymphocyte development in CD69 (-/-) mice. The number and phenotypic characteristics of T cells in different lymphoid tissues were normal, and the positive and negative selection of thymocytes was unaffected in the absence of CD69. T cells from CD69 (-/-) mice also showed normal proliferative capability and were able to provide apparently adequate help in antibody-specific responses. Furthermore, we have detected no alterations in the activation-induced cell death of T lymphocytes triggered by anti-CD3 mAb in CD69 (-/-) mice (Lauzurica et al, unpublished observations). These findings suggest that the function of CD69 in T cells is not essential or that it may be replaced by other molecules. It is also possible, however, that subtle defects in T-cell function may occur in CD69-deficient mice and that the assays employed in this study were not able to detect them.

There are several possibilities to explain the slight but significant increase in the humoral immune response against TD antigens observed in CD69 (-/-) mice. Immunoglobulin synthesis by B cells is under the control of different mechanisms, including those exerted by regulatory T cells. It is feasible that the absence of CD69 has subtle consequences on the immunoregulatory activities of T lymphocytes and that the increased synthesis of certain isotypes observed in CD69-deficient mice is related to defective modulation of B-cell function. Another possibility is that, as stated above, CD69 may exert a direct modulatory/inhibitory activity on certain cell subsets, including mature antibody-producing B lymphocytes. In this case, the absence of CD69 expression by antibody-producing cells may have as consequence a disregulation in isotype-specific immunoglobulin synthesis. Since only one ES cell line was used to generate the CD69 (-/-) mice, it is feasible, but highly unlikely, that the phenotype differences might be due to a defect in the ES cells at a locus linked to but different from CD69. Further studies are necessary to elucidate the precise mechanism of enhanced antibody production in CD69-deficient mice.

Although CD69 is expressed by platelets, myeloid precursors, activated neutrophils, and eosinophils, we found no abnormalities in these cells in CD69 (-/-) mice. Different mechanisms may account for these results, including (i) the activities previously reported for CD69 in these cells in vitro may not occur in vivo; (ii) because most CD69 functions have been defined in human cells, it is possible that mouse CD69 may not exert the same functions in different cells; and (iii) as stated above, CD69 function may be replaced by other molecules in CD69 (-/-) mice. The cloning has recently been reported of AICL, a gene with a highly similar sequence to that of CD69, although its cellular and tissue distribution at the protein level has not been described yet.[44] In addition, other yet uncharacterized molecules from the C-lectin NK cell complex may also participate, as well as different costimulatory molecules that have been extensively studied such as CD28.

In conclusion, CD69 is expressed by both B-cell precursors and activated B cells and seems to exert a subtle modulatory effect on B-cell development and antibody synthesis. This study also indicates that CD69 is not essential for TD T-lymphocyte development and cell proliferation. Nonetheless, subtle defects in some regulatory function of T cells cannot be ruled out. Functional redundancy of costimulatory molecules may account for the lack of gross abnormalities in the differentiation and activation of T cells in the absence of CD69. Because our CD69-deficient mouse line has been maintained in pathogen-free conditions, it is not known whether or not these mice exhibit increased susceptibility to some infectious agents; experiments to address this question are in progress. Other mouse lines deficient in immune response-related molecules (eg, IL-10, CCR-1, MIP-1α) are also apparently normal, but important abnormalities emerge when they are challenged with different pathological agents. Finally, the possible role of CD69 deficiency in autoimmune diseases deserves further research.

Acknowledgments

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The Generation of Mature, Single-Positive Thymocytes In Vivo Is Dysregulated by CD69 Blockade or Overexpression[1]

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During development in the thymus, mature CD4$^+$ or CD8$^+$ cells are derived from immature CD4$^+$CD8$^+$ cells through a series of selection events. One of the hallmarks of this maturation process is the expression of CD69, which first appears on thymocytes as they begin positive selection. We have used blockade and overexpression of CD69 to determine the role of CD69 in thymocyte development. Blockade of CD69 led to a reduction in single-positive cells and a concomitant increase in double-positive cells in the thymus. Overexpression of a CD69 transgene in the thymus resulted in a dramatic increase in both CD8SP and CD4SP cells. Coexpression with a TCR transgene demonstrated that both positive and negative selection were enhanced by the increased levels of CD69 on thymocytes. Finally, mice overexpressing CD69 displayed a sharp reduction in the number of T cells in the spleen and lymph node. Taken as a whole, these data suggest the involvement of CD69 in the process of selection and maturation during the trafficking of thymocytes to the medulla. *The Journal of Immunology,* **2002, 168: 87–94.**

The generation of functional T cells in the thymus requires an ordered sequence of events that is initiated by the rearrangement of the genes that encode the TCR chains. Cells that express functional TCRs are selected through a process that includes both positive and negative events. Positive selection allows for the further development of cells bearing TCRs that bind with low affinity to nonspecific peptides, while negative selection involves the deletion of those cells that express TCRs with high affinity for self-peptides (1–3). Concomitant with these developmental changes is the expression of a variety of cell surface markers. For example, an examination of the expression of the coreceptors CD4 and CD8 allows thymocytes to be subdivided into four broad cell populations. The earliest, least mature, of these populations lack expression of both CD4 and CD8 and are referred to as double-negative (DN)[4] thymocytes. It is within this population that TCR gene rearrangement begins. As these cells begin to undergo positive selection they coexpress CD4 and CD8 and become double-positive (DP) cells. As selection is completed, thymocytes express either CD4 or CD8 and become mature, single-positive (SP) thymocytes. These are the cells that emigrate from the thymus and populate peripheral lymphoid organs (4, 5).

In addition to the changes in the expression of cell surface markers, thymocytes traverse through the thymus as they develop. Immature thymocytes reside in the cortex, and as they begin the selection process they traffic from the cortex to the medulla. This transition is marked by the expression of other specific molecules. For example, the chemokine receptor CCR4 is up-regulated in thymocytes as they migrate from the cortex to the medulla (6–8). This in contrast to CCR9, which is expressed on both cortical and medullary thymocytes, but is down-modulated as thymocytes leave the thymus for the periphery (6, 8). These data suggest that chemokine/chemokine receptor interactions are involved in the migration of thymocyte subsets.

In addition to chemokine receptors, other cell surface molecules are expressed in a restricted fashion on developing thymocytes. One of these is the activation marker CD69, which is expressed at high levels on approximately 10–15% of thymocytes. Expression of CD69 is first seen on DP thymocytes as they begin positive selection, and experiments using TCR transgenic mice showed that only those thymocytes being selected express CD69 (9, 10). Several lines of evidence strongly suggest an active role for CD69 in TCR-mediated positive selection of thymocytes. All TCR$^+$ thymocytes are CD69$^+$, including both TCR^{low} DP cells and TCR^{high} SP cells (5). These CD69$^+$ cells can be further subdivided by expression of heat-stable Ag (HSA; HSA$^+$TCR^{low}, HSA$^+$TCR^{high}, and HSA$^-$TCR^{high}); however, CD69$^+$ cells do not express the mature T cell marker Qa-2 (5). Also, using an in vitro reaggregation system, Hare et al. (11) showed that CD69 may regulate an MHC-independent aspect of positive selection, suggesting that CD69 is not merely a marker for cells that have begun the selective process. Thus, CD69-expressing cells represent a population that is undergoing positive selection or has just completed that process.

Despite the compelling circumstantial evidence that CD69 is actively involved in thymocyte development and selection, there is no direct evidence. We have used overexpression and blockade of CD69 to directly test its role in thymocyte development, and demonstrate that CD69 plays a role in thymocyte selection.

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[4] Abbreviations used in this paper: DN, CD8CD4 double negative; DP, CD4CD8 double positive; SP, CD4 or CD8 single positive; HSA, heat-stable Ag.

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Materials and Methods

Abs and immunofluorescence

The Abs used in this study were as follows: anti-TCRαβ-FITC (H57-597), anti-CD3ε-FITC (145-2C11), anti-CD69-FITC (H1.2F3), anti-CD25-FITC (7D4), anti-CD8-APC (53-6.72), anti-CD4-PE (GK1.5), anti-CD44-FITC (KM114), anti-Vβ5-biotin (MR9-4), and anti-Qa-2-biotin (1-1-2) from BD PharMingen (La Jolla, CA); and anti-CD62L-FITC (MEL-14), anti-CD45RB-PE (16A), and anti-HSA-FITC (J11d) from Caltag Laboratories (Burlingame, CA). Clonotype anti-DO11.10 TCR (KJ1-26) was a gift from Dr. P. Marrack (National Jewish Hospital, Denver, CO).

For Ab staining, single-cell suspensions were prepared from freshly isolated lymphoid organs from control and experimental animals and suspended in PBS supplemented with 2% FCS and 0.1% sodium azide. In general, 10^6 cells were incubated on ice for 30 min with appropriate staining reagents as previously described (12). For direct staining, cells were first incubated with 2.4G2 (from ascites) to prevent nonspecific binding of mAbs via FcR interactions. Flow cytometric analysis was performed on either FACSCalibur or FACSvantage (BD Immunocytometry Systems, Mountain View, CA).

In vivo administration of Abs

Newborn C57BL/6 mice were treated daily for 7 days beginning at day 0 (within 24 h of birth) by the i.p. injection of 200 μg purified an anti-I-A^k mAb (10-2.16, BD PharMingen, as a negative control), an anti-I-A^b mAb (M5/114, BD PharMingen), or an anti-CD69 mAb (H1.2F3, BD PharMingen) suspended in PBS. In some experiments 2-wk-old C57BL/6 mice were treated daily for 7 days with 500 μg anti-CD69 (three mice per group) for 7 days. Then, the thymocytes were prepared and subjected to FCM analysis.

Generation of CD69 transgenic mice

CD69FL. A cDNA clone encoding the entire coding region of mouse CD69 (13) was subcloned into p1017, which contains the proximal mouse *lck* gene promoter and the human growth hormone gene (14). The transgene was excised with *Not*I and used for pronuclear injections.

CD69 Δcyt. A cDNA construct encoding a cytoplasmic-deletion mutant of mouse CD69 was generated by PCR using the primers 5′-agatctATGGAAGGATCCATTCAAGTT-3′ (amino terminus) and 5′-agatctTCATCTGGAGGGCTTGCTGCA-3′ (carboxyl terminus). The amino terminal primer begins, after the initiating ATG, at codon 34 of mouse CD69. The final construct contains three codons from the cytoplasmic domain of mouse CD69 (the initiating ATG and the final two amino acids of the cytoplasmic domain) preceding the membrane-spanning sequence and extracellular domain. The resulting product was cloned into p1017 and injected as described above.

Double-transgenic mice (CD69FL and either the OT-II or DO11.10 TCR transgene) were generated by crossing the appropriate single-transgenic lines and screening for the individual transgenes. In all cases transgene-positive mice were identified by PCR using primers to the human growth hormone gene as previously described (14).

Results

In vivo treatment with anti-CD69 mAb inhibits the generation of mature thymocytes

The up-regulation of CD69 expression during thymocyte selection suggests a role for CD69 in the generation of mature, SP thymocytes. To begin an analysis of the role of CD69 in thymocyte development, newborn C57BL/6 mice were injected daily for 1 wk with 200 μg anti-CD69 mAb. Control mice were injected with PBS, an irrelevant MHC class II Ab (anti-I-A^k), or the relevant MHC class II Ab (anti-I-A^b). The latter mice served as a control for the ability to block the generation of CD4SP cells. As shown in Fig. 1 the control mice displayed the anticipated phenotypes following Ab treatment: the anti-I-A^b-treated mice showed a decrease in the number of CD4SP cells relative to the PBS-treated mice, while the anti-I-A^k-treated mice showed no difference compared with the PBS controls (Fig. 1*A*). The effect of anti-I-A^b treatment was specific to CD4SP development as the number of CD8SP cells was unaffected.

The analysis of thymocyte development in the anti-CD69-treated mice showed reductions in both CD4SP (35–50%) and CD8SP (40–50%) cells, suggesting that blockade of CD69 inhibited the generation of SP cells (Fig. 1*A*). Further characterization of these mice showed that the numbers of Qa-$2^+$$HSA^-$ cells as well as $TCR\alpha\beta^+HSA^-$ cells, were reduced, consistent

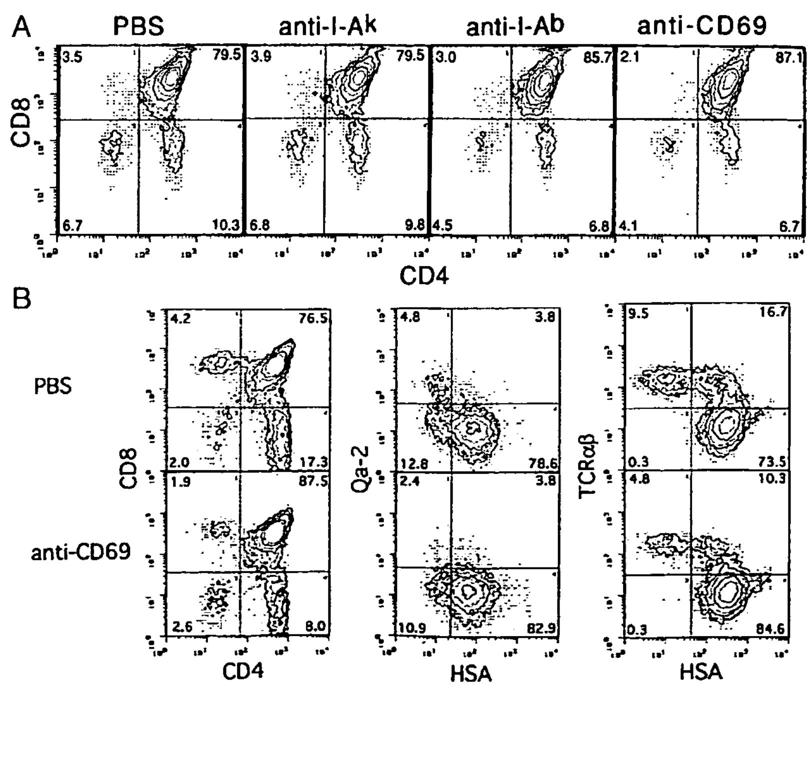

FIGURE 1. Inhibition of the generation of mature SP thymocytes by in vivo treatment with anti-CD69 mAb. *A*, Neonatal B6 mice (three mice per group) were treated daily for 7 days beginning on day 0 (within 24 h of birth) with 200 μg/ml of an anti-I-A^k mAb (10-2.16, as a negative control), an anti-I-A^b mAb (M5/114), or an anti-CD69 (H1.2F3) suspended in PBS. Thymocytes were individually prepared from the treated mice and were subjected to flow cytometric analysis with anti-CD4-FITC and anti-CD8-APC. Representative CD4/CD8 profiles are shown with the percentage of cells in each quadrant. Cellularity (average of three mice analyzed for each treatment): PBS, 81 × 10^6; I-A^k, 86 × 10^6; I-A^b, 77 × 10^6; CD69, 81 × 10^6. B, Two-week-old B6 mice were treated daily with 500 μg anti-CD69 (three mice per group) for 7 days, and the thymocytes were individually prepared and stained with the indicated Abs. Representative profiles of CD4/CD8, HSA/Qa-2, and HSA/TCRαβ are shown with the percentage of cells in each quadrant.

with a reduction of SP thymocytes (Fig. 1*B*). In all cases thymic cellularity was unchanged, suggesting that the reduction seen in anti-CD69-treated mice was not due to nonspecific lysis of CD69-expressing cells.

Overexpression of CD69 in the thymus results in an increase in SP thymocytes

The data presented above support a role for CD69 in the generation of SP thymocytes. We next determined the effect of CD69 overexpression on thymocyte development. A cDNA encoding full-length mouse CD69 was expressed from the *lck*-proximal promoter in transgenic animals (these mice will be referred to as CD69FL). Several founder lines were established that expressed varying levels of CD69 in the thymus, as judged by cell surface expression.

As shown in Table I, increased levels of CD69 expression in the thymus tended to skew the developmental profile seen in these mice, with the lines expressing the highest levels of CD69 showing increased numbers of CD4SP and CD8SP cells. Fig. 2 shows the analysis of a representative line, CD69FL-1. Examination of CD69 expression in this line showed that it was markedly increased relative to that in littermate controls, with approximately 99% of thymocytes expressing CD69 (Fig. 2*A*). When CD3 levels on thymocytes from CD69FL-1 and NLC mice were analyzed, the CD69FL-1 line displayed a higher percentage of $CD3^{high}$ cells (Fig. 2*B*). Also, there appeared to be two populations of $CD3^{high}$ cells in CD69FL-1 mice, with a small shoulder of cells with slightly lower CD3 levels and a second population with higher CD3 levels (also see Fig. 4). The peak representing the highest level of CD3 expression was CD4SP (gate 1) cells, while the lower peak contained CD8SP cells (gate 2). This pattern of CD3 expression is similar to that seen when CD3 levels are analyzed on mature splenic T cells. We next examined the CD4/CD8 profile of CD69FL-1 and NLC mice. There was a dramatic increase in the number of CD4SP and CD8SP cells and a diminution of CD4CD8DP cells in CD69FL-1 mice (Fig. 2*C*), with approximately 37% of the thymocytes from CD69FL-1 mice showing an SP phenotype compared with 17% for the NLC. There was a concomitant decrease in DP cells in CD69Fl-1 mice, and overall thymic cellularity was comparable between the two sets of mice, suggesting that an increase in total cell numbers cannot account for this difference in SP cells. We have now examined four CD69FL

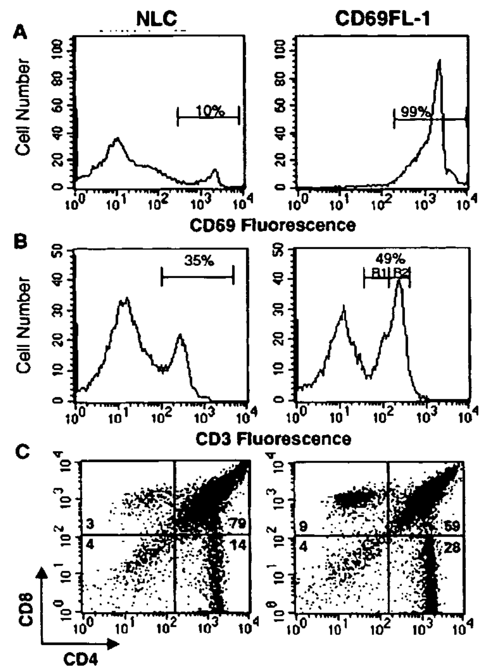

FIGURE 2. Analysis of thymocyte development in CD69FL mice. Thymi from CD69FL-1 and NLC mice were harvested, and cells were analyzed by flow cytometry for CD69 expression (*A*), CD3 expression (*B*), and CD4/CD8 expression (*C*). *A* and *B*, The numbers refer to the percentage of cells in the indicated gate. *B*, Gates R1 and R2 represent two populations of $CD3^+$ cells, with R1 predominantly $CD8^+$ and R2 predominantly $CD4^+$. *C*, The numbers refer to the percentage of cells in each quadrant. The data are representative of eight CD69FL-1 and eight NLC mice analyzed.

lines, and all show a similar phenotype, with the number of SP thymocytes proportional to the level of CD69 expression (number of SP cells varies from 25 to 58%; data not shown).

Additional evidence as to the phenotype of the thymocytes from the CD69FL mice came from an analysis of other cell surface markers, including HSA, $CD45^{RB}$, Qa-2, and CD62L on SP cells. Fig. 3 shows the expression of these markers on CD4SP cells from

Table I. *Thymic phenotype of CD69FL transgenic mouse lines*

Strain[a]	Cellularity[b]	% $CD69^+$/MFI[c]	% CD4 SP	% CD8 SP	% CD4CD8 DP
CD69FL-1					
CD69 Tg^+	93	99/1430	28	9	59
NLC	95	10/360	14	3	79
CD69FL-2					
CD69 Tg^+	53	89/620	26	19	51
NLC	70	16/95	10	3	86
CD69FL-3					
CD69 Tg^+	80	95/1125	21	14	62
NLC	82	16/117	10	2.5	81
CD69FL-4					
CD69 Tg^+	43	26/103	16	8	76
NLC	65	20/95	10	5	85
CD69FL-5					
CD69 Tg^+	80	98/723	25	20	50
NLC	80	13/26	9	3	82

[a] Each strain represents an individual CD69FL transgenic line. Normal littermate control (NLC) in each case was age- and sex-matched with CD69FL line (CD69 Tg^+).
[b] Total thymic cellularity, $\times 10^6$.
[c] Percent $CD69^+$ thymocytes/Mean fluorescence intensity for the cells in the $CD69^-$ gate.

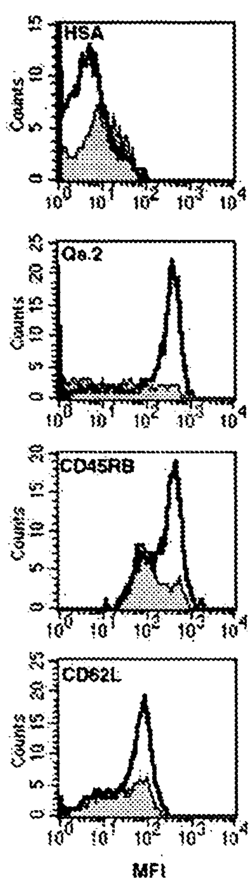

FIGURE 3. CD4SP cells from CD69FL-1 mice have a mature phenotype. Thymocytes from CD69FL-1 and NLC mice were harvested, and cells were analyzed by three-color flow cytometry for CD4, CD8, and HSA, Qa.2, $CD45^{RB}$, or CD62 ligand expression. CD4SP cells were gated, and the expression of the indicated marker was determined. The shaded histogram represents cells from NLC mouse, and the open histogram represents cells from CD69FL-1. Data are representative of four CD69Fl-1 and four NLC mice analyzed.

CD69FL-1 and NLC mice. CD4SPs from the control animals had an $HSA^{+}Qa\text{-}2^{-}CD45RB^{low}CD62L^{low}$ phenotype. In contrast, the cell surface phenotype of the CD4SP cells from CD69FL-1 mice more closely resembles that of mature, peripheral T cells ($HSA^{-}Qa\text{-}2^{+}CD45RB^{high}CD62L^{high}$). The CD8SP cells displayed a similar phenotype (data not shown). Finally, thymocytes from the CD69 transgenic mice have a higher proliferative capacity, compared with control mice, when stimulated through the TCR (data not shown). Taken as a whole, these data demonstrate that overexpression of full-length CD69 in the thymus results in the accumulation of SP cells in the thymus that more closely resemble mature, peripheral T cells then SP thymocytes.

Thymocyte development in mice expressing a cytoplasmic deletion mutant of CD69 resembles that seen in mice expressing full-length CD69

To determine whether the phenotype seen in the CD69-overexpressing transgenic animals required CD69-mediated signal transduction, we generated mice expressing a cytoplasmically truncated form of CD69 from the *lck*-proximal promoter (see *Materials and Methods* for details of the construct). Several founder lines were generated, and T cell development was studied in four lines (CD69Δcyt-1–4) that displayed CD69 levels roughly equivalent to those seen in the CD69FL lines (Figs. 2*A* and 4*A* and data not shown). As was seen with CD69FL-1 mice, CD69Δcyt-1 and littermate control mice displayed similar thymic cellularity. Thymocyte development in CD69Δcyt mice was also very similar to that seen in CD69FL-1 mice. For example, the CD69Δcyt-1 line showed the two populations of $CD3^{high}$ cells seen in CD69FL mice (Figs. 2*B* and 4*B*), as well as increases in both CD4SP and CD8SP cells (Fig. 4*C*), although the CD4SP/CD8SP ratio in CD69Dcyt mice differed from that in CD69FL-1 mice. The significance of this last finding is unclear at this time. Thus, the ability of CD69 to affect the DP to SP transition does not necessarily require a signal through CD69. This suggests that overexpression of the extracellular domain of CD69 is the major cause of the phenotypes seen in the CD69FL and CD69Δcyt transgenic mice, possibly by affecting interactions with its ligand. However, the severity of the phenotype seen in CD69FL mice was greater than that seen in CD69Δcyt mice, demonstrating a possible role for CD69-mediated signals.

Positive and negative selection in CD69FL mice

A possible explanation for the phenotype seen in CD69FL-1 mice is that overexpression of CD69 leads to enhanced positive selection, and thus an increase in the number of SP cells. To directly study the role of CD69 on the positive and negative selection of thymocytes, we crossed CD69FL-1 mice with two TCR transgenic mouse lines, OT-II and DO11.10. Both TCRs are specific for the same chicken OVA peptide ($OVA_{323-332}$) (15). OT-II-expressing T cells recognize this peptide presented in the context of $I\text{-}A^{b}$, while DO11.10-expressing T cells see it presented in the context of $I\text{-}A^{d}$. However, T cells expressing the DO11.10 TCR also are alloreactive against $I\text{-}A^{b}$ (16), and thymocytes bearing the transgenic

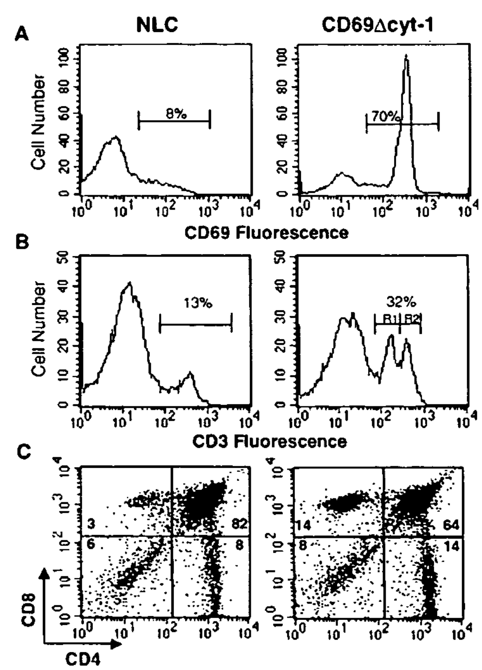

FIGURE 4. Analysis of thymocyte development in CD69Δcyt mice. Thymi from CD69Δcyt-1 and NLC mice were harvested, and cells were analyzed by flow cytometry for CD69 expression (*A*), CD3 expression (*B*), and CD4/CD8 expression (*C*). *A* and *B*, The numbers refer to the percentage of cells in the indicated gate. *B*, Gates R1 and R2 represent two populations of $CD3^{+}$ cells, with R1 predominantly $CD8^{+}$ and R2 predominantly $CD4^{+}$. *C*, The numbers refer to the percentage of cells in each quadrant. Data are representative of five CD69Δcyt-1 and five NLC mice analyzed. Cellularity: NLC, 50×10^{6}; CD69Δcyt-1, 54×10^{6}.

TCR are negatively selected in H-2^b mice (17). Thus, both positive and negative thymocyte selection in CD69FL-1 mice, which are on the C57BL/6 background, can be examined using these two TCR transgenic lines.

To generate CD69FL/OT-II double-transgenic mice, we crossed the CD69FL-1 line with C57BL/6 mice expressing the OT-II TCR transgene (these mice will be referred to as OT-II). There was no significant difference in thymic cellularity between the two strains (Fig. 5). Thymocyte development was studied in OT-II and CD69FL-1/OT-II F$_1$ mice, using Abs against CD4, CD8, CD3, and Vβ5 (the β-chain of the transgenic TCR). As shown in Fig. 5, the OT-II mice showed a skewing toward CD4SP cells, reflecting positive selection of the OT-II TCR-expressing thymocytes. CD69/OT-II mice displayed a greater skewing toward the development of CDSP cells, with greater than 60% of thymocytes CD4$^+$/Vβ5$^+$ (Fig. 5). We have also examined the ability of CD4SP cells from these mice to respond to Ag in a proliferation assay. CD4$^+$ cells were purified from OT-II and CD69/OT-II mice and cultured with irradiated C57BL/6 splenocytes in the presence of the antigenic peptide. No difference was found between OT-II- and CD69/OT-II-derived cells (data not shown). Taken as a whole, these data show that the process of positive selection proceeds at an enhanced rate in mice overexpressing CD69 in the thymus, and that the selected CD4SP cells respond normally to antigenic stimulation.

We next analyzed negative selection in mice overexpressing CD69. To do this, we took advantage of the fact that thymocytes expressing the DO11.10 TCR as a transgene undergo negative selection on the H-2^b background. CD69FL/DO11.10 TCR double-transgenic mice were generated by crossing the CD69Fl-1 transgenic line with C57BL/6 mice expressing DO11.10 TCR (these mice are referred to as DO11). Thymocyte development was assessed, using flow cytometry, in F$_1$ mice from this cross that represented the four relevant genotypes (−/−, −/DO11 CD69/−, and CD69/DO11). Thymocytes from the control lines (−/− and CD69/−) showed the same pattern of development as those shown in Fig. 2, with CD69FL-expressing mice showing an increase in SP cells and a concomitant decrease in DP cells (data not shown).

An analysis of DO11 and CD69/D011 mice is shown in Fig. 6. DO11 mice displayed a decrease in thymus cellularity (29 × 10^6 for DO11, compared with 156 × 10^6 for NLC mice) along with a marked increase in DN cells and a decrease in DP cells.

Initial examination of CD69/DO11 mice showed that they also had a dramatic decrease in thymic cellularity, reduced approximately 83% (27.5 × 10^6 compared with 156 × 10^6 for the NLC mouse). Interestingly, the double-transgenic mice displayed features of each single transgene animal, showing both increased SP cells and increased DN cells. In fact, the increase in DN cells was more dramatic than that seen in DO11.10 TCR mice (32% DN cells in CD69/DO11 mice vs 16% in DO11 mice). Also, as was seen in mice expressing either CD69FL or CD69Δcyt, mice expressing both the DO11.10 TCR transgene and the CD69Δcyt transgene displayed the same overall thymic phenotype as CD69/DO11 mice (data not shown).

We next examined the expression of DO11.10 TCR in these mice. As shown in Fig. 6, both lines had similar numbers of CD3$^+$ cells. When the expression of DO11.10 TCR was examined using the clonotypic Ab KJ1-26 (17), both DO11 and CD69/DO11 mice showed a reduction in the number of clonotype-positive cells. DO11.10 TCR expression was then examined in individual thymic subpopulations of both sets of animals. CD4SP cells from both DO11 and CD69/DO11 mice showed a dramatic reduction in KJ1-26 staining, suggesting that the clonotype-positive cells had been negatively selected. However, as mentioned above, CD69/DO11 mice had more CD4SP cells than DO11 mice (Fig. 6). These cells do not express the transgenic TCR and most likely arise through rearrangement of endogenous TCRα genes. This finding is consistent with the increased accumulation of CD4SP cells in CD69FL mice (Fig. 2).

We next examined the expression of the transgenic TCR in the DN compartment in both mice. A majority of DN cells in DO11 mice expressed CD3 and were KJ1-26$^+$ (Fig. 6). Earlier work had shown that these KJ1-26$^+$, CD4$^-$CD8$^-$ cells represented a lineage that bypassed the DP stage (16). In contrast, most of the DN cells from the CD69/DO11 mice were CD3$^-$ and KJ1-26$^-$.

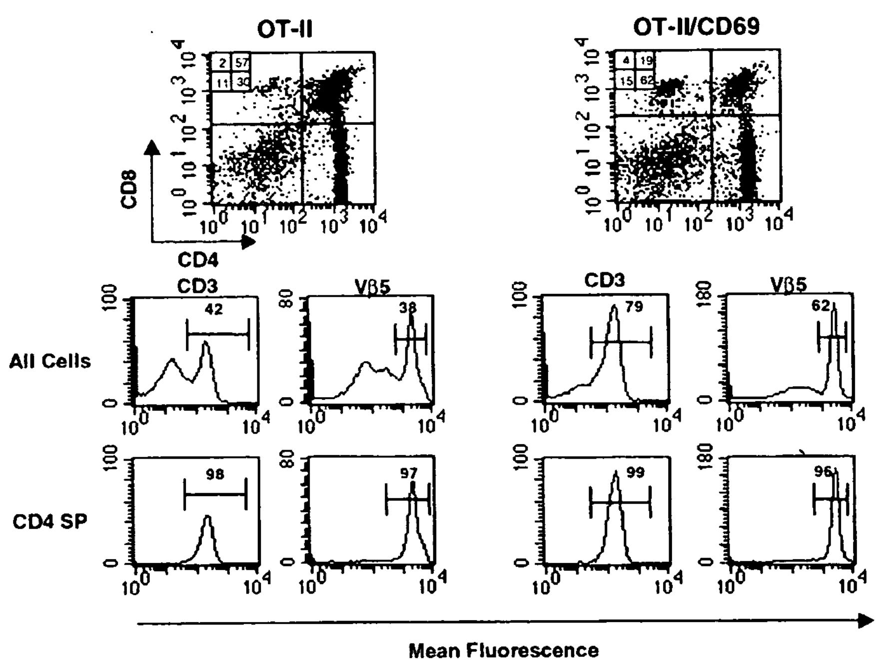

FIGURE 5. Positive selection in CD69FL transgenic mice. CD69FL-1 and OT-II TCR transgenic mice were bred. Cells were isolated from thymi from the indicated mouse lines and analyzed by three-color flow cytometry for CD4 and CD8, and either CD3 or Vβ-5 expression. *Top panel*, CD4/CD8 profiles from each mouse line. Numbers in the *upper corner* represent the percentage of cells in that quadrant. *Bottom panel*, CD3 vs Vβ-5 expression in SP cells from thymi isolated from OT-II and CD69/OT-II mice. Levels of CD3 and Vβ-5 expression were determined for the indicated cell populations for these two mouse lines. The number refers to the percentage of cells in the gate. Cellularity: OT-II, 42 × 10^6; CD69/OT-II, 48 × 10^6.

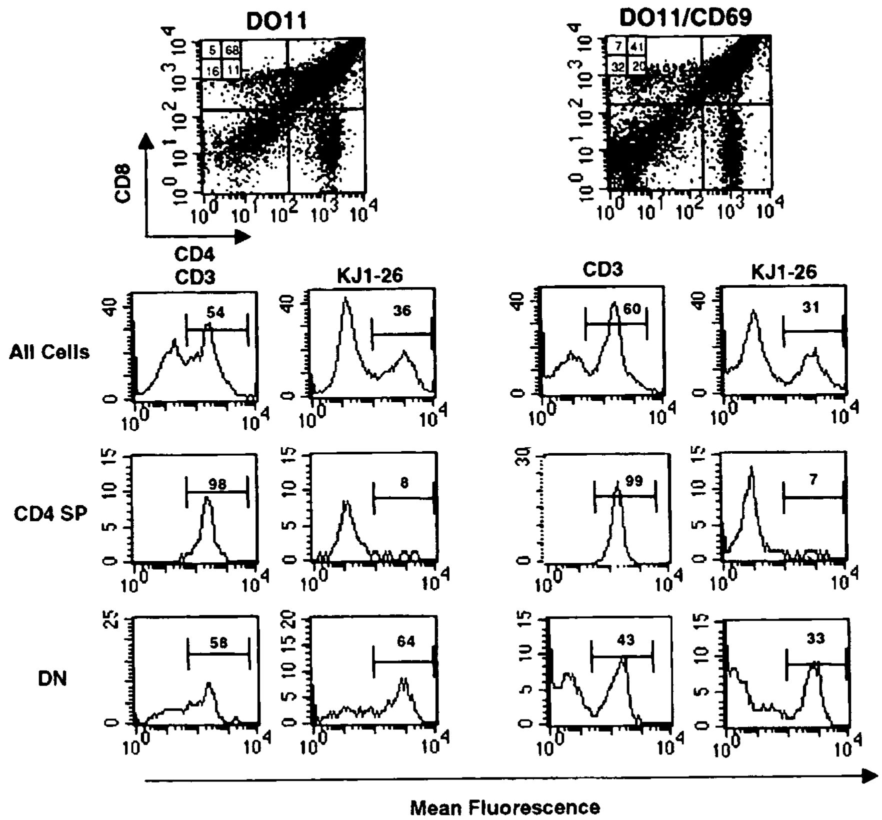

FIGURE 6. Coexpression of CD69FL and DO11.10 TCR leads to an increase in negative selection. Cells were isolated from thymi from the indicated mouse lines and analyzed by three-color flow cytometry for CD4, CD8, and either CD3 or KJ1-26 expression. *Top panel*, CD4/CD8 profiles from each mouse line. The numbers in the *upper left corner* represent the percentage of cells in each quadrant. *Bottom panel*, CD3 vs KJ1-26 expression in subpopulations from thymi of DO11 and CD69/DO11 mice. Levels of CD3 and KJ1-26 expression were determined on cells from the indicated cell populations for these two lines. The number refers to the percentage of cells in the gate. Cellularity: DO11, 29 × 10^6; CD69/DO11, 28 × 10^6.

Mice expressing CD69 transgenes had reduced T cells in peripheral lymphoid organs

As shown above, thymi from mice that overexpress CD69, either full-length or containing a cytoplasmic truncation, displayed a dramatic increase in SP cells (Figs. 2 and 4). These cells had the cell surface characteristics of mature T cells ($TCR^{high}Qa\text{-}2^{+}HSA^{-}$; Fig. 3 and data not shown). One possible explanation for this phenotype is that following maturation the SP cells are incapable of exiting the thymus. If this explanation were correct, one prediction would be a reduction in the number of peripheral T cells. We tested this hypothesis by examining CD3 levels and CD4/CD8 profiles of spleens and lymph nodes isolated from CD69FL and CD69Δcyt mice. It was apparent that the spleens and lymph nodes from both sets of animals were significantly smaller than those in the control animals, and this observation was supported by CD3 and CD4/CD8 profiles from each animal. As shown in Fig. 7*A*, CD69FL-1 mice had dramatically reduced numbers of T cells in spleen and almost no T cells in lymph node (data not shown). Concomitantly, the number of $CD3^{+}$ cells was severely reduced (data not shown). We also examined T cells in spleens and lymph nodes of CD69Δcyt animals. Similar to what we observed in CD69FL-1 animals, spleens and lymph nodes in CD69Δcyt-1 were smaller than those in littermate control mice. However, CD69Δcyt-1 mice had a 50% reduction of peripheral T cells number (Fig. 7*B*), a less severe loss than what was seen in CD69FL-1 mice. This finding was consistent with the less severe thymic phenotype seen in these mice (Fig. 4) and suggests a role for CD69 signaling in the phenotypes seen in these two sets of mice.

We also examined peripheral T cells in OT-II and CD69/OT-II mice (Fig. 7*C*). Splenic cellularity was comparable in the two mice. OT-II mice showed an increase in $CD4^{+}$ cells in the spleen, nearly all of which were $V\beta5^{+}$ (data not shown). However, spleens from CD69/OT-II mice were nearly devoid of T cells. Subsequent analysis determined that the cellularity in the spleen in these animals was maintained by an increase in the number of B cells (data not shown). The reduction in the number of peripheral T cells in OT-II/CD69 mice was greater than that in CD69FL-1 mice.

Discussion

A great deal of progress has been made defining the role of TCR selection in T cell development in the thymus (reviewed in Refs. 1–3). However, little is known about the roles of cell surface molecules, such as CD69, whose expression is also regulated during thymocyte development. In this report, we have shown that CD69 plays an active role in the development of thymocytes. We have shown that blockade of CD69, through in vivo Ab administration, inhibits development at discreet stages. Ab blockade of CD69 caused a reduction in the number of SP thymocytes and a concomitant increase in the number of DP cells (Fig. 1). While these data strongly suggest that blockade of CD69 inhibits SP development, we cannot rule out that cross-linking CD69 with the mAb results in the deletion of SP thymocytes, or that the lower levels of SP cells reflects phagocytosis of Ab-coated cells. To address the effect of CD69 blockade more directly, we have generated transgenic mice expressing a secreted form of the CD69 extracellular domain. A preliminary analysis of thymocyte development in these mice has suggested that they are similar to what was observed in Ab-treated animals, supporting the hypothesis that CD69 blockade inhibits the development of SP thymocytes (data not shown).

In contrast to what was observed in mice in which CD69 interactions were blocked, mice that overexpressed CD69 in the thymus displayed a dramatic increase in the numbers of CD4SP and CD8SP cells (Figs. 2 and 4 and Table I). In addition, these cells had the phenotype of mature T cells in that they were $TCR^{high}Qa$-

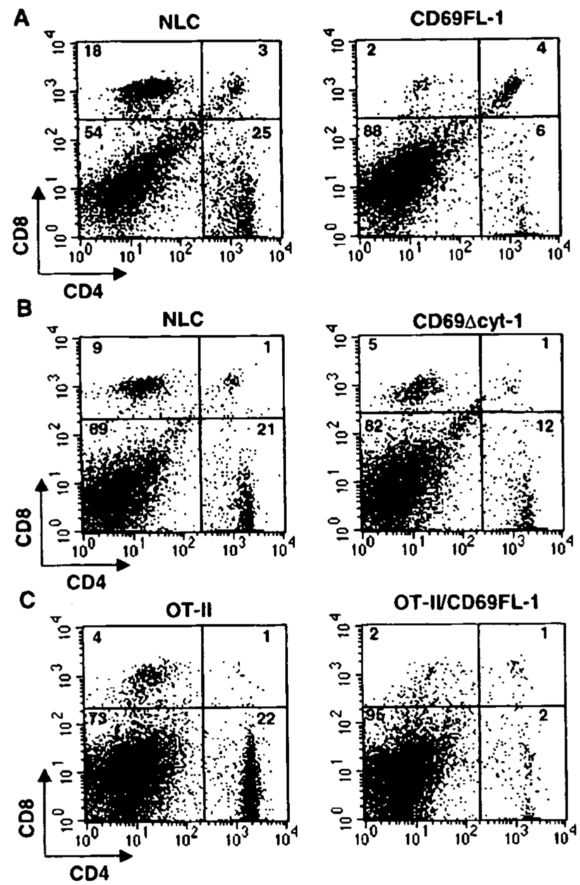

FIGURE 7. Decrease in peripheral T cell numbers in CD69FL (*A*), CD69Δcyt (*B*), and OT-II/CD69 (*C*) mice. Spleens were harvested from CD69FL, CD69Δcyt, OT-II/CD69, and NLC mice and analyzed for CD4 and CD8 expression by flow cytometry. The percentage of cells in each quadrant is indicated. Total cell numbers were: CD69FL-1/NLC, 109/92 × 10^6; CD69Δcyt/NLC, 80/78 × 10^6; CD69-OT-II/OT-II, 65/50 × 10^6.

2$^+$HSA$^-$. This is the phenotype seen on T cells as they leave the thymus and seed the periphery (5). The fact that this population is present in large numbers in the thymus of CD69 transgenic mice, but not in littermate control mice, suggests that it is the continued expression of CD69 that affects the developmental profile of these cells. Consistent with a more mature phenotype, CD4SP cells from CD69FL mice were more responsive to TCR engagement (D. J. Kasprowicz and S. F. Ziegler, unpublished observation).

The accumulation of SP cells, at the expense of DP cells, in CD69FL mice suggests differences in thymic selection between these animals. We tested this hypothesis by examining the role of CD69 in the selection events using mice expressing transgenic TCRs that were either positively or negatively selected on the C57BL/6 background. The data generated from these double-transgenic mice supports the hypothesis that overall thymic selection was more efficient in mice overexpressing CD69 (Figs. 5 and 6). For example, double-transgenic mice expressing either positively or negatively selected TCRs had similar cellularity as their single TCR transgenic littermates. The positively selecting mice (CD69/OT-II) had twice as many CD4SP cells as the OT-II mice, all of which expressed the TCR transgene (Fig. 5, *bottom panel*). The negatively selecting mice (CD69/DO11) also displayed an increase in CD4SP cells relative to the single TCR transgenic line. However, these CD4SP cells did not express the transgenic TCR (as shown by staining with KJ1-26; Fig. 6, *bottom panel*), suggesting that they arose from cells that rearranged endogenous TCRα genes. Those thymocytes expressing the DO11.10 TCR were deleted. These data suggest that, similar to the CD69FL-1 mice, thymocytes in both sets of double transgenic mice were being driven to the SP stage. In the CD69/OT-II mice, these SP cells were selected and accumulated. In CD69/DO11.10 mice those thymocytes that expressed the TCR transgene were deleted, and those that were able to rearrange and express endogenous TCR α-chains accumulated. Consistent with this was our finding that the spleens of DO11 mice contained CD4$^-$CD8$^-$DO11.10$^+$ cells, as has been previously reported when this TCR transgene is expressed on a H-2^b background (16). In CD69/DO11 mice these cells were not present in the periphery, and these mice, similar to CD69FL-1, had a dramatic reduction in the number of peripheral T cells (data not shown).

To assess whether CD69 signal transduction was involved in thymocyte development, we generated mice overexpressing a cytoplasmic mutant of CD69 (CD69Δcyt). In cell culture studies we have shown that expression of this construct in cells inhibited the signaling of endogenous CD69 (data not shown). An analysis of thymocyte development in these mice showed that cell-surface expression of CD69 is sufficient to lead to an increase in SP cells. However, the phenotype in mice overexpressing functional CD69 (CD69FL) is more dramatic than that in mice expressing an equivalent level of CD69Δcyt (compare Figs. 2 and 4). Again, similar to the CD69FL mice, the phenotypes seen in mice expressing the CD69Δcyt transgene correlated with levels of transgene expression. These data, taken together, suggest a model by which a combination of CD69 interaction with an as yet unknown ligand, coupled with CD69-mediated signal transduction, contributes to the generation of SP thymocytes.

There are several possible explanations for the accumulation of thymic SP cells in the CD69 transgenic mice. One possible explanation is that there is greater turnover of those SP cells in the thymus. The fact that overall thymic cellularity is unchanged in these mice argues against this. Also, we have begun to examine apoptosis in these mice, using annexin V staining and TUNEL analysis and found no difference between NLC and CD69 transgenic mice (D. J. Kasprowicz and S. F. Ziegler, unpublished observations). Another possible explanation is that CD69 is involved in the trafficking of thymocytes during their maturation. In this model, unregulated expression of CD69 on thymocytes increases the movement of thymocytes from the cortex to the medulla during differentiation. The inability to down-regulate CD69 expression on these cells causes them to remain in the thymus. Support for this model comes from indirect immunofluorescence of human thymus using anti-CD69 mAb. In this study Jung et al. (18) found that only scattered cells in the cortex expressed CD69, and these cells tended to cluster in the subcapsular region. These may correspond to the CD44$^+$CD25$^-$ DN population that we have shown expresses CD69 in mouse thymus (T. Nakayama and S. F. Ziegler, unpublished observation). In contrast, most, if not all, medullary thymocytes were CD69$^+$. These data are consistent with a model in which CD69 acts as a trafficking molecule, perhaps acting in concert with CCR4, for cells migrating from the cortex to the medulla during selection. As these cells complete their developmental program they down-regulate CD69 and leave the thymus to seed peripheral lymphoid organs. Overexpression of CD69 on the surface of thymocytes would therefore be predicted to lead to an increase in mature thymocytes and a lack of T cells in peripheral organs. This is the phenotype observed in CD69FL transgenic mice and, to a lesser extent, in CD69Δcyt mice (Figs. 2, 4, and 7).

The finding the mice lacking CD69 have normal thymic development (T. Nakayama, D. J. Kasprowicz, M. Yamashita, and S. F. Ziegler, unpublished results) suggests that there are additional molecules expressed on thymocytes capable of interacting with the same ligand and generating related signals. We have searched the public EST databases and have found three novel C-type lectins that are expressed in the thymus (data not shown). We are currently testing the roles of these molecules in thymocyte development. Also, as mentioned above, our interpretation of the data is predicated on the existence of a ligand for CD69 that is expressed in the thymus. Using purified soluble CD69 we have detected specific binding on cell lines that can serve as APC (data not shown). These data are consistent with a CD69 binding partner being expressed in thymus by cells that regulate selection. We are currently examining thymic stromal cell lines for solCD69 binding.

Acknowledgments

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CD69 is an immunoregulatory molecule induced following activation

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CD69 is an early leukocyte activation molecule expressed at sites of chronic inflammation. The precise role of CD69 in immunity has not been elucidated owing to the absence of a known ligand and adequate *in vivo* models to study its physiological function. Although previous *in vitro* studies suggest that CD69 is an activatory molecule in different leukocyte subsets, recent studies in CD69-deficient mice have revealed a non-redundant role for this receptor in downregulation of the immune response through the production of the pleiotropic cytokine transforming growth factor-β (TGF-β). The possible cellular and molecular mechanisms of action of this molecule are discussed herein.

Introduction

Self-limitation of the immune response is crucial to its control and molecules induced during lymphocyte activation might act as negative regulators. In this Opinion, we discuss recent results that identify CD69 as a potential negative regulator. CD69 is an early membrane receptor transiently expressed on lymphocyte activation, not detected in resting lymphocytes, and selectively expressed in chronic inflammatory infiltrates and at the sites of active immune responses *in vivo*. Although early *in vitro* data suggested that CD69 exerts a proinflammatory function, recent *in vivo* results indicate that this receptor might act as a regulatory molecule, modulating the inflammatory response. In addition, CD69 might act specifically on an as yet uncharacterized T-cell regulatory subset. These recent insights provide a novel view of the function of this receptor, even though a full picture of the spatial and temporal regulation of the immune response by CD69 will require detailed characterization of its ligand(s).

Early data: CD69 exerts a co-stimulatory effect *in vitro*

The *CD69* gene is located within the natural killer (NK) gene complex on mouse chromosome 6 and human chromosome 12 [1,2] and codes for a type II C-type lectin ascribed to the family of NK receptors. CD69 is expressed following activation in all bone marrow-derived cells except erythrocytes (reviewed in Ref. [3]). Most NK lectin receptors directly mediate their activatory or inhibitory effects through their cytoplasmic domains [4]. However, the cytoplasmic domain of CD69 is short and lacks any identifiable function-associated motifs. Thus, no signalling proteins have been described that directly associate with its cytoplasmic domain, although recent results show that CD69 activates Syk in a Src-dependent manner in NK cells [5]. These tyrosine kinases control downstream activation of phospholipase Cγ2 (PLCγ2) and Vav1 that, in turn, activate the Rac–ERK (extracellular signal-regulated protein kinase) pathway [6], which is implicated in NK-cell activation. In addition, some studies have reported the involvement of a CD69-coupled heterotrimeric G protein in its intracellular signalling pathway [7–9].

The rapid and transient induction of CD69 expression on T cells suggests that it might enhance activation and/or differentiation, as occurs with CD40L (CD154) or CD25. In the absence of a known ligand, *in vitro* studies to dissect the possible function of CD69 were based on the use of specific monoclonal antibodies (mAbs) (reviewed in Ref. [3]). In the presence of phorbol esters, anti-CD69 mAbs stimulate the production of interleukin-2 (IL-2), which increases T-cell proliferation [10,11], and tumour necrosis factor-α (TNF-α) synthesis [9,12], whereas they induce nitric oxide (NO) secretion by monocytes [13] and activation of arachidonic acid metabolism and degranulation in platelets [14], suggesting that CD69 could act as a proinflammatory receptor. In addition, cross-linking of CD69 with secondary antibodies mediates early signalling events, such as extracellular Ca^{2+} influx [9–11], relieving the blockade in capacitative calcium entry in antigen-primed T cells [8]. Moreover, antibodies against CD69 significantly inhibit the ability of T cells to activate macrophages by cell contact [15], suggesting that a putative CD69 counter-receptor expressed by macrophages is involved in the production of proinflammatory cytokines. Therefore, CD69 can apparently mediate immune cell activation and exert proinflammatory effects *in vitro* either directly or indirectly. However, CD69 engagement also triggers apoptosis in different cell types, such as monocytes or eosinophils [16,17], and might mediate inhibitory signals on IL-1 receptor (IL-1R)- or CD3-mediated T-cell proliferation [18]. All these data indicate that CD69 behaves *in vitro* more as a co-stimulatory receptor than as a net inhibitory or activatory molecule, although the fate of this co-stimulation could vary depending on the cellular context.

Recent insights: immunoregulatory role of CD69

The *in vivo* models initially chosen for the study of CD69 function were based on its pattern of expression. Studies

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in CD69-transgenic mice focused on thymic selection [19,20], a process in which CD69 expression is transiently induced (Box 1). Despite the *in vitro* evidence suggesting a possible proinflammatory role for CD69, constitutive expression of CD69 by T cells in transgenic mice is not associated with inflammatory conditions [19,20]. Furthermore, analysis of antigen-specific responses in mice has not revealed reduced T-cell activation in the absence of CD69 [21], suggesting that this receptor does not exert a net positive co-stimulatory effect in T cells *in vivo*, although a redundant role as a positive co-stimulus for T cells cannot be ruled out.

Given the somewhat contradictory *in vitro* and *in vivo* results, it became appropriate to study the role of CD69 in an *in vivo* model of chronic inflammation. This analysis was based on two lines of evidence. First, CD69 is persistently expressed at inflammatory foci [22]. Second, the *CD69* gene is located at the Cia3 trait loci on rat chromosome 4 and mouse chromosome 6 [1], syntenic to human 12p12–p13 [2], a region that contains susceptibility loci for several autoimmune diseases, including collagen induced arthritis (CIA) [23,24]. Remarkably, the study of CIA in CD69-deficient mice unveiled a new regulatory role for CD69 (Figure 1). CD69-deficient mice develop an exacerbated form of CIA with higher T- and B-cell responses against collagen [25]. This hyper-responsiveness correlates with reduced levels of TGF-β in inflamed joints (Figure 1). TGF-β acts as an anti-inflammatory cytokine in CIA [26], and treatment with blocking anti-TGF-β antibodies exacerbates arthritis severity, increasing proinflammatory cytokines and chemokines, in wildtype but not in CD69-deficient mice [25]. The reduced levels of TGF-β and the absence of CD69 could be causally associated. In this regard, CD69 cross-linking *in vitro* promotes TGF-β synthesis [25,27]. In addition, TGF-β synthesis is dependent on ERK activation [28] and CD69 cross-linking mediates ERK activation [6]. Hence, the regulatory effects of CD69 *in vivo* appear to be mediated through the synthesis of a pleiotropic cytokine, which might be finely tuned by the controlled expression of CD69 ligand(s).

CD69 cross-linking induces TGF-β production in $CD4^+$ and $CD8^+$ T cells as well as in NK cells and macrophages [25,27], suggesting that this receptor exerts a wide immunoregulatory action, and that other cells, expressing the corresponding counter-receptor, might also participate in this phenomenon. Thus, CD69 could influence not only adaptive but also innate immunity. Accordingly, in an NK-sensitive tumour model in mice, CD69 deficiency leads to reduced TGF-β synthesis by immune cells that results in a high production of chemokines, with decreased lymphocyte apoptosis, accumulation of NK cells and enhanced tumour lysis [27]. Supporting these data, blockade of TGF-β signalling in T cells enhances anti-tumour immunity by facilitating the expansion of tumour-specific $CD8^+$ T cells [29].

Both the NK-sensitive tumour model and the CIA model demonstrate that CD69 deficiency leads to diminished TGF-β levels that support an enhanced immune response, resulting in a more efficient depletion of tumours or increased inflammation in the CIA model [25,27]. The use of an antibody that downregulates CD69 expression *in vivo* reproduced in wildtype mice the phenotype found in CD69-deficient mice [27], further supporting the proposed immunoregulatory role of CD69.

However, as mentioned earlier, CD69 cross-linking *in vitro* also mediates production of proinflammatory mediators [9,12–14], thus suggesting that CD69 could have a dual role, mediating the synthesis of different cytokines, depending on the particular cellular context. It has been reported that CD69-deficient mice are resistant to the induction of granulocyte-mediated acute arthritis, which is initiated by the administration of exogenous anti-collagen II antibodies and endotoxin [30], an inflammatory condition in which the regulatory mechanisms exerted by lymphocytes are not involved. It is feasible that although TGF-β has a predominant inhibitory effect on T and B cells [31,32], this cytokine might act as a chemotactic and activating agent on granulocytes [33], which are the main mediators of this acute model of arthritis. Therefore, reduced synthesis of TGF-β might still account for the attenuated inflammatory response seen in this model. Alternatively, CD69 might affect the synthesis of proinflammatory molecules in a non-redundant way in this particular model, whereas in the CIA model any effect of CD69 on the synthesis of proinflammatory molecules [15] could be compensated by other receptors. The possible dual role of CD69 makes it difficult to predict the final outcome of engagement by its ligand(s),

Box 1. CD69 and thymocyte physiology *in vivo*

CD69 is transiently expressed in thymocytes that are undergoing positive selection or that have just completed this process [47–49]. Positive selection is a multi-stage process involving a first step in which CD69 is induced in double positive thymocytes, a process dependent on MHC molecules expressed by thymic epithelial cells, and a second step to maturation to single positive thymocytes that is MHC-independent [50]. These data indicate that CD69 is a marker of a thymocyte subset that differentiates and proliferates in an MHC-independent fashion and suggest that this molecule mediates this process. However, CD69 deficiency does not affect thymic development and positive or negative selection of thymocytes [21]. By contrast, the constitutive expression of CD69 during T-cell development induces an increase in both CD8 and CD4 single positive thymocytes in thymus medulla [19,20]. These data suggest that the constitutive expression of CD69 does not interfere with thymocyte development but inhibits the export of mature single positive thymocytes to the periphery [19]. By crossing CD69 transgenic mice with different TCR transgenic mice, Nakayama *et al.* showed enhanced negative selection that caused a reduction in the number of T cells in peripheral lymphoid organs [20]. Interestingly, CD69Δcyt transgenic mice, constitutively expressing CD69 without the cytoplasmic domain, show a phenotype similar to CD69 transgenic mice [20], suggesting that the putative CD69 ligand expressed in the thymus is responsible for this phenotype when CD69 is overexpressed. Because CD69-deficient mice show normal thymocyte selection [21], either the putative CD69 thymic ligand acts just as a mechanism of retention of $CD69^+$ thymocytes or the signal for thymocyte selection induced through CD69L is triggered by molecules other than CD69 that are expressed by thymocytes.

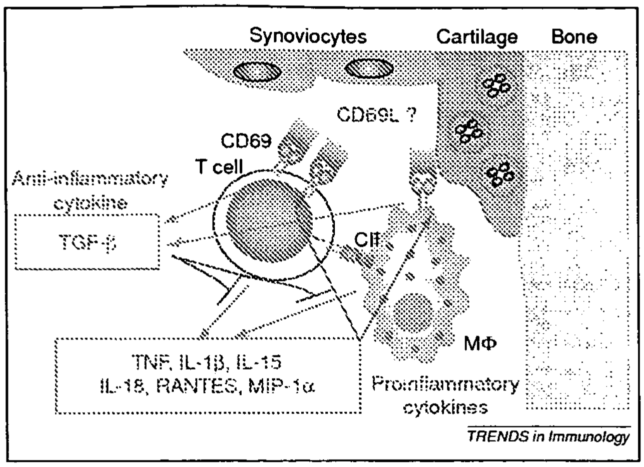

Figure 1. CD69 acts as an immunoregulatory molecule through the production of TGF-β. In collagen-induced arthritis, T cells are activated by collagen-derived peptides (CII) presented by macrophages (Mϕ), which induce CD69 expression and the release of proinflammatory cytokines. These cytokines and co-stimulatory molecules contribute to the persistent expression of CD69 in the inflammatory foci. Under such circumstances, the interaction of CD69 with its putative ligand(s) (CD69L) would induce the synthesis of the anti-inflammatory cytokine TGF-β, which reduces the secretion of proinflammatory cytokines and the activation of immune cells, thereby ameliorating tissue damage.

which might result in a pro- or anti-inflammatory state, conditioned by the particular environment.

Possible regulatory steps affected by CD69

Recent results indicate that CD69 modulates the synthesis of immunoregulatory molecules. Initial T-cell activation and antigen-driven T-cell proliferation are not affected by the absence of CD69 [21]. However, CD69 might affect the immune response during T-cell differentiation (Figure 2), involving immunoregulatory cytokines that include, but might not be limited to, TGF-β, which controls T-cell differentiation [31] and that, depending on the stimulation provided, could also regulate proinflammatory molecules.

CD69 is persistently expressed *in vivo* by T cells under certain conditions characterized by chronic inflammation [22], and *in vitro* on constant stimulation with proinflammatory cytokines or through certain adhesion receptors [15,34]. As stated earlier, the CIA model in CD69-deficient mice shows that local TGF-β levels in the joint are reduced [25], suggesting that in wildtype mice this receptor would interact with its putative ligand(s), inducing TGF-β, and thus dampen the local immune response (Figure 1). In this regard, TGF-β is found in the synovial fluid from rheumatoid arthritis (RA) patients [35], where it might counterbalance the activity of proinflammatory cytokines. In addition, the presence of IgG anti-CD69 autoantibodies, detected in the serum of a subset of RA patients, correlates with disease severity [36]. Therefore, we can hypothesize that these autoantibodies are able to block the interaction of CD69 with its putative ligands, decreasing TGF-β production and resulting in more severe disease. Alternatively, these autoantibodies could enhance signalling through CD69, generating proinflammatory mediators.

The induction of TGF-β synthesis through CD69 ligation might also take place in lymph nodes during the antigen-induced T-cell differentiation (Figure 2). In this regard, it is well known that CD69 is transiently expressed during T-cell activation and differentiation following antigen presentation by dendritic cells (DCs), and it is feasible that CD69 could exert a negative regulatory activity through TGF-β production. TGF-β downregulates antigen-presenting cell (APC) function [37,38] and limits activation and polarization of T cells to a Th1 or Th2 phenotype [31] (Figure 2). Therefore, enhanced T-cell differentiation in the absence of CD69 could explain the stronger effector response [25]. This negative regulatory role for every CD69-expressing T cell would normally lead to a non-specific limitation of the immune response. However, CD69 might mediate such role only in specific circumstances, for example, CD69 might be upregulated under tolerogenic conditions in the absence of other activating molecules that might counteract the negative effect of CD69. Alternatively, the expression of CD69 ligands under tolerogenic but not immunogenic conditions could limit the regulatory function exerted through CD69. Finally, other cell types with a variety of functional subsets, including monocytes and DCs, might express CD69, leading to distinct outcomes depending on the specific cellular and pathophysiological setting in which CD69 is expressed.

CD69 and regulatory T cells

T regulatory (Treg) cells have an impaired capacity to respond to proliferative signals and are able to inhibit other immune cell functions through cell–cell contact or through the production of anti-inflammatory cytokines, such as TGF-β, IL-10 or IL-4 [39,40]. Natural Treg cells are generated in the thymus and are characterized by their high expression of CD25, which suppresses effector responses through cell–cell contact in a cytokine-independent manner. However, adaptive Treg cells are generated from mature T lymphocytes after antigenic stimulation in the periphery, show a variable expression of CD25, and their mechanism of suppression of effector T-cell responses is cytokine-dependent [41]. It is feasible that CD69 could be persistently expressed by a subset of these cells (Figure 2).

In a murine lupus model, a subset of $CD4^+CD69^+$ cells has been detected in peripheral lymphoid tissues and inflammatory infiltrates. These cells are anergic and unable to synthesize proinflammatory cytokines [42]. Moreover, these $CD4^+CD69^+$ cells inhibit cytokine synthesis by $CD4^+CD69^-$ cells in a process that seems to be dependent on TGF-β because it is inhibited by anti-TGF-β antibodies [42]. Interestingly, peripheral blood mononuclear cells from lupus patients show an increased expression of CD69 [43] and the poor *in vitro* response of these cells to different stimuli is well known. Likewise, freshly isolated human synovial fluid T cells display a profound state of hypo-responsiveness that correlates with the expression of CD69 [44]. Therefore, some T cells bearing CD69 appear to possess the two main characteristics of Treg cells, namely their anergic behavior and their regulatory role. However, it is not currently known whether only a subset of the entire population of CD69-expressing cells is able to synthesize TGF-β *in vivo* and acts as a regulatory cell subset in different chronic

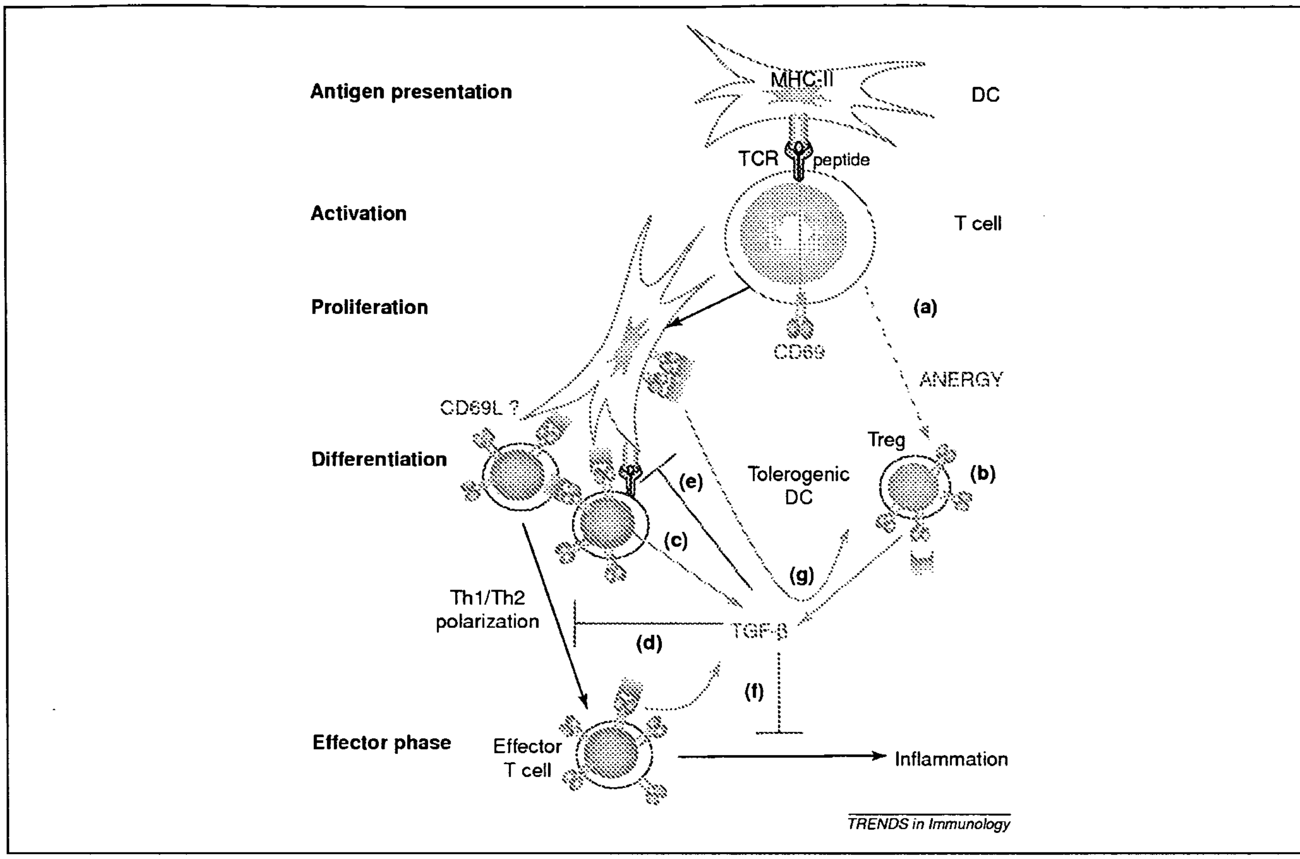

Figure 2. CD69 might be involved in multiple regulatory steps during the immune response. The antigen-specific signal activates T cells through the TCR and induces CD69. (a) However, incomplete activation might lead to anergy. (b) Some of these anergic lymphocytes might act as Treg cells and CD69 could act through TGF-β production as a survival factor for this cell subset. Alternatively, if the balance of the second signal is positive, T lymphocytes will proceed to cell proliferation. In this case, CD69 might exert its immunoregulatory effect at two different levels. First, if CD69L were expressed by DCs at lymph nodes, (c) $CD69^+$ T cells might produce TGF-β, a cytokine that inhibits both (d) T-cell differentiation (Th1 and Th2 polarization) and (e) APC function. (f) Second, if CD69L is expressed at inflammatory cell infiltrates, leukocytes persistently expressing high levels of CD69 would produce TGF-β, which would dampen inflammation. (g) Finally, CD69 expression might define a subset of tolerogenic DCs.

inflammatory conditions [42–44]. It is expected that CD69 should have an essential role in the proper function of these cells. TGF-β induces FoxP3 and a regulatory phenotype in TCR-challenged $CD4^+CD25^-$ naïve T cells [45] and this might also explain that, under certain circumstances, the CD69-dependent induction of TGF-β could influence the development of a subset of adaptive Treg cells.

Concluding remarks

Previous results *in vitro* pointed to CD69 as a stimulatory receptor, however, recent results *in vivo* have shown that the behaviour of CD69 is more complex. The absence of CD69 leads to an enhanced immune response in two independent models: increased severity of a T-cell driven animal arthritis model [25] and augmented rejection of NK-sensitive tumours [27]. CD69 mediates TGF-β production and the effect of this pleiotropic cytokine might account for the regulatory effect of CD69, although other mediators could be involved. CD69 could affect different steps in the distinct mechanisms responsible for the limitation of immune responses. First, CD69 might have a role in the deletion of lymphocytes by apoptosis following activation [27]. Second, during antigen presentation, partial activation signals might be able to simultaneously induce CD69 expression [15,34] and an anergic state, which is a characteristic of CD69-expressing lymphocytes in chronic inflammatory diseases [42,44]. Conceivably, these anergic lymphocytes could correspond to an as yet poorly defined adaptive Treg-cell subset that could act as a bystander suppressor lymphocyte population through the production of TGF-β or other immunoregulatory cytokines, mediating localized or systemic immune deviation [42,46]. Third, CD69 engagement might regulate the final balance of Th1/Th2 differentiation. The characterization of CD69 ligand(s), and the knowledge of their spatial and temporal expression, will shed further light on the precise immunoregulatory functions of CD69.

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Increased expression of pro-inflammatory cytokines and metalloproteinase-1 by TGF-β1 in synovial fibroblasts from rheumatoid arthritis and normal individuals

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SUMMARY

Transforming growth factor (TGF)-β1 is expressed abundantly in the rheumatoid synovium. In this study, the inflammatory effect of TGF-β1 in rheumatoid arthritis (RA) was investigated using cultured fibroblast-like synoviocytes (FLS) from RA and osteoarthritis (OA) patients, as well as non-arthritic individuals. mRNA expressions of IL-1β, tumour necrosis factor (TNF)-α, IL-8, macrophage inflammatory protein (MIP)-1α and metalloproteinase (MMP)-1 were increased in RA and OA FLS by TGF-β1 treatment, but not in non-arthritic FLS. Enhanced protein expression of IL-1β, IL-8 and MMP-1 was also observed in RA FLS. Moreover, TGF-β1 showed a synergistic effect in increasing protein expression of IL-1β and matrix metalloproteinase (MMP)-1 with TNFα and IL-1β, respectively. Biological activity of IL-1 determined by mouse thymocyte proliferation assay was also enhanced by 50% in response to TGF-β1 in the culture supernatant of RA FLS. DNA binding activities of nuclear factor (NF)-κB and activator protein (AP)-1 were shown to increase by TGF-β1 as well. These results suggest that TGF-β1 contributes for the progression of inflammation and joint destruction in RA, and this effect is specific for the arthritic synovial fibroblasts.

Keywords IL-1β IL-8 MIP-1α rheumatoid arthritis TGF-β1 TNFα metalloproteinase-1

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic and systemic inflammatory disease characterized by progressive destruction of joints. Hypertrophic RA synovial tissue is comprised of large numbers of infiltrating inflammatory cells and excessively proliferating synovial lining cells which produce several inflammatory as well as anti-inflammatory cytokines. Analyses of the cytokine mRNA and proteins in the RA synovial tissue revealed that TNFα, IL-1, IL-6, GM-CSF and TGF-β were expressed at high levels in RA patients [1–3]. Among these cytokines, TNFα and IL-1β are known to be the pivotal pro-inflammatory cytokines in the pathogenesis of RA. However, the role of other cytokines in RA has not yet been fully understood.

The presence of TGF-β1 at a high level in the RA synovium suggests that TGF-β1 *per se* or in combination with other cytokines plays an important role in the progression of RA. Although TGF-β1 is well known for its immune-suppressive and anti-inflammatory properties, it is also capable of promoting inflammation [4]. In a RA animal model, injections of TGF-β into the synovium induced an inflammatory response with accumulation of neutrophils, and exacerbated arthritic responses [5,6]. Moreover, anti-TGF-β antibody blocked accumulation of inflammatory cells and tissue pathology in an experimental model of chronic erosive polyarthritis [4].

Although it is not clearly understood, there are several ways by which TGF-β can regulate RA pathogenesis. First, TGF-β can modulate expression of inflammatory cytokines such as TNFα and IL-1β [7]. Secondly, production and activity of metalloproteinases are regulated by TGF-β [8–10]. Thirdly, TGF-β1 is strongly chemotactic and may attract inflammatory cells to synovial tissue [4,6]. Fourthly, synovial hypertrophy can be accelerated by TGF-β since it induces proliferation of fibroblasts [7] and may also modulate apoptosis of synovial fibroblasts. Finally, VEGF is strongly induced by TGF-β, and therefore, TGF-β can contribute indirectly to angiogenesis in arthritic synovium [11].

In the present study, we have investigated the effect of TGF-β1 on the expression of inflammatory cytokines and MMP-1 in RA FLS. The results indicated that TGF-β1 induced or increased the expressions of IL-1β, TNFα, IL-8, MIP-1α and MMP-1, and synergized with other proinflammatory cytokines in RA FLS. These effects of TGF-β1 were similar in RA and osteoarthritis (OA) FLS, but not in non-arthritic FLS.

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MATERIALS AND METHODS

Primary culture of human FLS and cytokine treatment

Synovial tissues were obtained from RA and OA patients during the total joint replacement surgery. RA and OA were diagnosed according to the criteria of the American College of Rheumatology [12,13]. Non-arthritic synovial tissues were obtained from the knee joint of two trauma patients undergoing arthroscopic examination and the unaffected knee joint of a sarcoma patient undergoing amputation. The synovial tissues were minced and digested with 500 units/ml of type II collagenase (Sigma, St Louis, MO, USA) and 3 mg/ml of dispase (grade II) (Boehringer Mannheim, Indianapolis, IN, USA) in MEM by shaking vigorously at 37°C for 30 min. Supernatant containing the released cells was removed and the digestion procedure was repeated four times. Isolated cells were cultured in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) containing 15% FBS and antibiotics (100 μg/ml streptomycin, 100 units/ml penicillin G, and 0·25 μg amphotericin B). When the cells had grown to confluence, they were split at a 1 : 2 ratio. FLS were used for experiments at passages 4–10. TGF-β1 was purchased from R&D systems (Minneapolis, MN, USA) and TNFα and IL-1β from Biosource (Camarillo, CA, USA). Cytokines (TGF-β1, TNFα or IL-1β) were added to the cultures to a final concentration of 10 ng/ml.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from synovial cells as described previously [14]. Reverse transcription was performed using oligo(dT)$_{17}$ primer (Bioneer, Taejun, Korea) and Molony murine leukaemia virus (M-MuLV) reverse transcriptase (Gibco BRL, Grand Island, NY, USA) at 37°C for 1 h cDNA synthesized from total RNA (0·5 μg unless otherwise indicated) was amplified with a specific primer pair in a 25-μl reaction mixture. PCR primers for IL-1β and TNFα were purchased from Clontech (Palo Alto, CA, USA) and PCR was carried out according to the manufacturer's instruction. Sequences of other PCR primers were as follows: IL-8 forward, 5′atg-act-tcc-aag-ctg-gcc-gtg 3′; IL-8 reverse, 5′tta-tga-att-ctc-agc-cct-ctt-caa-aaa-ctt-ctc 3′; MIP-1α forward, 5′cgc-ctg-ctg-ctt-cag-cta-cac 3′; MIP-1α reverse, 5′ tgt-gga-ggt-cac-acg-cat-gtt 3′; MMP-1 forward, 5′gca-cag-ctt-tcc-tcc-act 3′; and MMP-1 reverse, 5′cat-ccc-ctc-caa-tac-ctg 3′. The thermocycling programmes consisted of 30 cycles at 94°C for 1 min, 60°C 1 min, and 72°C 1 min for IL-8 and MIP-1α, and at 94°C 1 min, 52°C 1 min, and 72°C 1 min for MMP-1. As a negative control, RT-PCR was performed in parallel without a template. mRNA from RA synovial tissue or RA FLS stimulated with TNFα or IL-1β was used as a positive control.

Immunoblotting

Cells treated with TGF-β1 were lysed in 1x lysis buffer (31·25 mM Tris-HCl, pH 6·8, 10% glycerol, 1% SDS, 2·5% β-mercaptoethanol) and heated for 10 min at 100°C. The cell lysate (80 μg of protein) was subjected to electrophoresis on a 10% polyacrylamide gel and the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). The membrane was incubated with a primary antibody for 1 h followed by a horseradish peroxide-conjugated secondary antibody for 1 h at room temperature. Bands of interest were detected by enhanced chemiluminescence (ECL) (Amersham, Buckinghamshire, UK) according to the manufacturer's instruction. Antibody for IL-1β was purchased from R&D systems (Minneapolis, MN, USA) and antibodies for IL-8 and MIP-1α from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while antibody for MMP-1 was from Oncogene (Cambridge, MA, USA). Densitometry of the data was carried out using LabWorks software produced by UVP Inc. (Cambridge, UK).

Biological activity assay of IL-1

Biological activity of IL-1 in the culture supernatant was determined by mouse thymocyte proliferation assay in triplicate. FLS were incubated in serum-depleted RPMI-1640 for 12 h, and further incubated in RPMI-1640 containing 10 ng/ml of TGF-β1 and 0·2% FBS at 37°C for 72 h, and the culture supernatant was harvested. Thymocytes were obtained from 6–8-week-old female DBA2 mice (Korean Chemical Institute, Taejun, Korea). Culture supernatant (50 μl) was added to thymocytes (2×10^6 cells in 150 μl) in RPMI-1640 : F_{12} (1 : 1) medium containing 5% FBS, 1 mM glutamine, antibiotics, 50 μM β-mercaptoethanol, 0·7 μg/ml concanavalin A (Con A) and 10 μg/ml polymixin B (Sigma, St Louis, MO, USA). Thymocytes were cultured for 30 h before adding [^{3}H]thymidine (1 μCi/well). After 18 h, thymocytes were harvested onto a glass fibre filter and [^{3}H]thymidine incorporation was measured by liquid scintillation counting.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from cells treated with TGF-β1. The oligonucleotide with the NF-κB or AP-1 binding consensus sequence (Promega, Madison, WI, USA) was end-labelled with [γ-^{32}P]dATP (Amersham, Buckinghamshire, UK) using T4 DNA polynucleotide kinase. Labelled oligonucleotide probes were incubated with nuclear extracts (3 μg of proteins) in the binding buffer [50 mM HEPES, pH 7·5, 0·5 mM EDTA, 0·5 mM DTT, 1% NP-40, 5% glycerol, 50 mM NaCl, 1 μg of poly(dI:dC)] at room temperature for 40 min. For the cold competition experiment, unlabelled oligonucleotide in 100-fold molar excess was added to the binding reaction. For supershift, antibodies specific for p65 or Fos (Santa Cruz, Santa Cruz, CA, USA) were incubated with the nuclear extract for 30 min at 4°C before the labelled oligonucleotide was added. The samples were electrophoresed on a 6% native polyacrylamide gel in 0·5 × TBE buffer, and the gels were dried and visualized by autoradiography.

RESULTS

mRNA expression of IL-1β and TNFα in response to TGF-β1

The cultured synovial cells were fibroblast-like: CD68$^-$, CD64$^-$, CD14$^-$, HLA-DQ$^+$ and HLA-DR$^-$ (data not shown). TGF-β1 mRNA expression was readily detected not only in RA and OA, but also in non-arthritic FLS (data not shown). The effect of TGF-β1 on expression of the major proinflammatory cytokines, IL-1β and TNFα, was assessed in these cells at 2–4 h post-stimulation. Competitive PCR showed that the level of IL-1β mRNA increased by 25–50-fold by TGF-β1 treatment in RA FLS (Fig. 1a). In non-arthritic FLS and MRC5, the basal levels of IL-1β mRNA were >10 and >100 times higher than that in RA FLS, respectively. However, IL-1β expression did not change by TGF-β1 treatment in non-arthritic FLS (NS1), and only mildly increased (by 2·5-fold) in MRC-5. We analysed additional FLS cultures by RT-PCR and confirmed enhanced expression of IL-1β

mRNA by TGF-$\beta 1$ stimulation in 4 RA FLS (Fig. 1b). Three OA FLS showed similar results as RA. Two additional nonarthritic FLS did not increase IL-1β expression upon TGF-$\beta 1$ stimulation, again confirming the competitive PCR results.

Expression of TNFα mRNA was also shown to increase significantly in RA and OA FLS (Fig. 1c). In non-arthritic FLS, the TNFα expression was only mildly increased. TNFα-treated FLS was used as positive controls for RT-PCR and no bands were observed in negative controls where no templates were added (data not shown).

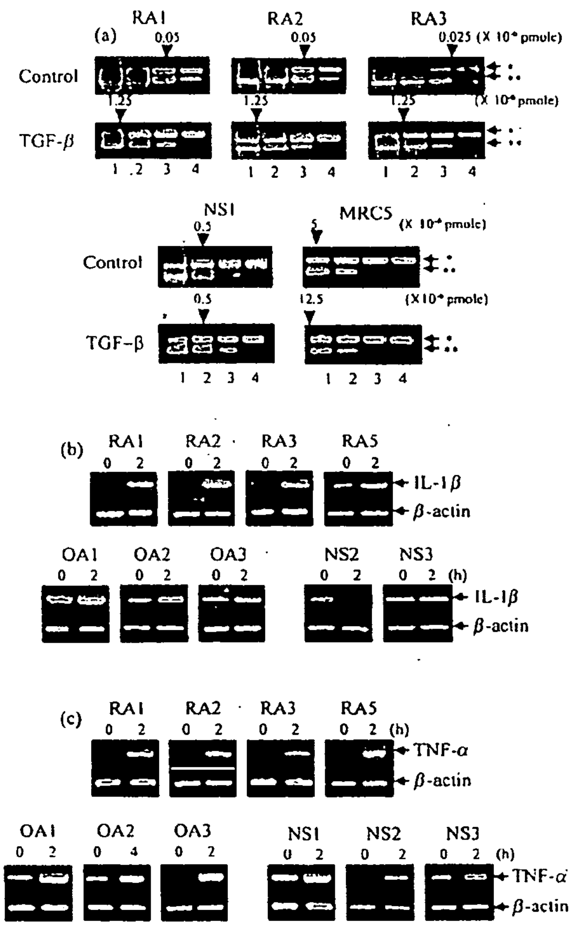

Fig. 1. Effect of TGF-$\beta 1$ on the mRNA expression of IL-1β. (a) IL-1β mRNA expression was analysed quantitatively by competitive PCR: Three RA and one non-arthritic FLS cultures as well as MRC-5 were treated with TGF-$\beta 1$ for 2 h. cDNA synthesized from the total RNA and the PCR competitor in a 10-fold serial dilution were co-amplified with IL-1β primers. The amount of the IL-1β competitor ranged from 5×10^{-4} pmole (lane 1) to 5×10^{-7} pmole (lane 4). The upper bands (*) are products amplified from IL-1β cDNA and the lower bands (**) are products from the competitor. Arrowheads indicate where the intensities of upper and lower bands are equal. (b) IL-1β expression was analysed in additional RA, OA and non-arthritic FLS stimulated with TGF-$\beta 1$ for 2 h. (c) Effect of TGF-$\beta 1$ on the mRNA expression of TNFα. TGF-$\beta 1$ treatment and RT-PCR were carried out as described in (b), except that the amplification was performed for 35 cycles.

Expression and secretion of IL-1β in response to TGF-β1

To assess changes in IL-1β protein expression, immunoblot analysis was carried out in RA5 FLS. In the absence of brefeldin A (BFA), induction of IL-1β protein by TGF-$\beta 1$ was not detectable, whereas that by TNFα could be observed (Fig. 2a). In the presence of BFA, however, the 35 kDa precursor form of IL-1β was observed in RA FLS treated with TGF-$\beta 1$ (Fig. 2a, lane 5), suggesting that the IL-1β protein induced by TGF-$\beta 1$ was mostly secreted. A synergistic effect between TGF-$\beta 1$ and TNFα on IL-1β expression was also found (Fig. 2b). IL-1 bioactivity was measured in triplicate by thymocyte proliferation assay. In the culture supernatant of RA FLS treated with TGF-$\beta 1$ for 72 h, the bioactivity of IL-1 was enhanced by 50% compared to that

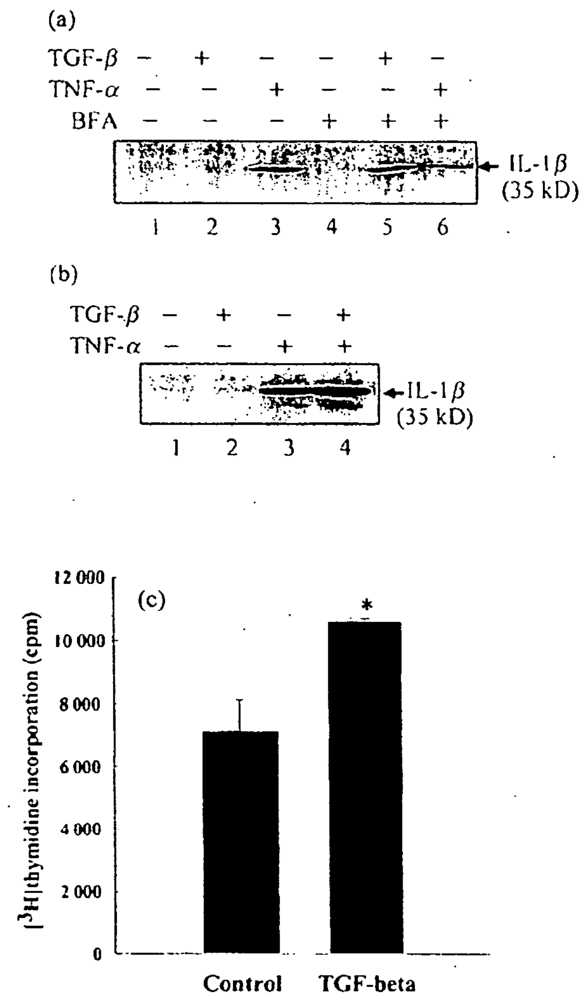

Fig. 2. Effect of TGF-$\beta 1$ on the expression of IL-1β protein in RA FLS. (a) RA5 FLS were stimulated with TGF-$\beta 1$ (10 ng/ml) or TNFα (10 ng/ml) for 12 h in the absence (lanes 1–3) or presence (lanes 4–6) of BFA (0·5 μg/ml). IL-1β protein was analysed by immunoblotting using IL-1β specific antibody. (b) RA FLS were stimulated with TGF-$\beta 1$ and/or TNFα for 24 h and the IL-1β protein expression was analysed by immunoblotting. (c) Bioactivity of IL-1 in the culture supernatant of RA2 FLS was measured by thymocyte proliferation assay in triplicate. Error bars represent standard deviation. $^{*}P \leq 0{\cdot}05$ (Student's t-test).

in the control supernatant ($P \leq 0{\cdot}05$, Student's *t*-test) (Fig. 2c). This experiment was repeated four times and similar results were obtained.

IL-8 and MIP-1α expression in response to TGF-β1

TGF-β1 is a strong chemoattractant for monocytes and neutrophils [7]. However, as yet it has not been shown clearly whether TGF-β induces expression of other chemokines. Therefore, the TGF-β1 effect on expression of IL-8, a prototype CXC chemokine and MIP-1α, a CC chemokine, was examined. Similar to its effect on IL-1β expression, TGF-β1 enhanced the level of IL-8 mRNA in RA and OA, but not in the non-arthritic FLS (Fig. 3a). TNFα-treated FLS was used as a positive control for RT-PCR. The IL-8 protein expression in RA FLS was also markedly induced (Fig. 3b). The level of IL-8 protein induced by TGF-β1 was comparable to that induced by TNF-α. As for the IL-1β, increased expression of IL-8 protein by TGF-β1 was revealed only after BFA treatment. The MIP-1α mRNA expression was also elevated in both RA and OA FLS (Fig. 3c). Again, in non-arthritic FLS, TGF-β1 did not augment MIP-1α mRNA expression. Despite the increase in the steady state mRNA level, the protein expression of MIP-1α was not induced by TGF-β1 in RA FLS (data not shown).

MMP-1 expression in response to TGF-β1

MMP-1 (interstitial collagenase, collagenase-1) plays an important role in destruction of joints in arthritis. The effect of TGF-β on MMP-1 expression has been contradictory. We showed here that TGF-β1 increased MMP-1 expression in RA and OA FLS (Fig. 4). Expression of MMP-1 mRNA reached to a peak at 2–4 h post-stimulation and started to decline after 12 h. In OA FLS, MMP-1 expression was also increased by TGF-β1, but with a slower rate (Fig. 4a). However, in non-arthritic FLS, the mRNA level of MMP-1 did not change. Similar results were obtained in additional RA, OA and non-arthritic FLS cultures (Fig. 4b). For a positive control of MMP-1 RT-PCR, IL-1β treated RA FLS was used (data not shown). Protein expression of MMP-1 was also enhanced in RA FLS by TGF-β1 stimulation (Fig. 4c). There was a remarkable synergism between TGF-β1 and IL-1β for the protein expression of MMP-1, suggesting the importance of TGF-β1 in inducing MMP-1 expression *in vivo*. In OA FLS, TGF-β1 significantly enhanced MMP-1 expression induced by IL-1β. However, TGF-β1 did not increase the MMP-1 protein expression either alone or together with IL-1β in non-arthritic FLS (Fig. 4c).

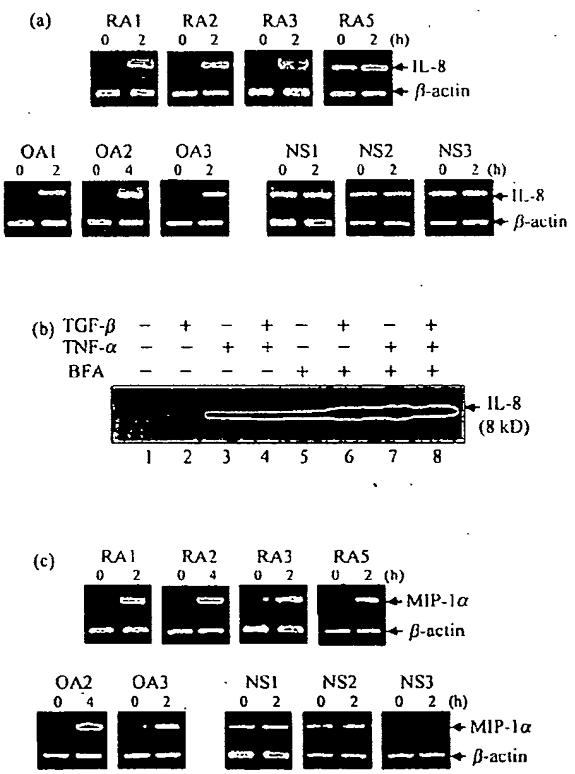

Fig. 3. Effect of TGF-β1 on IL-8 and MIP-1α expression. (a) IL-8 mRNA expression was analysed in RA, OA and non-arthritic FLS treated with TGF-β1 for 2 or 4 h. RT-PCR was performed as described in Materials and methods. (b) IL-8 protein expression was analysed by immunoblotting using anti-IL-8 antibody. RA5 FLS were stimulated with TGF-β and/or TNFα for 12 h in the absence (lanes 1–4) or presence (lanes 5–8) of BFA (0·5 μg/ml). (c) MIP-1α mRNA expression was analysed by RT-PCR as in (a).

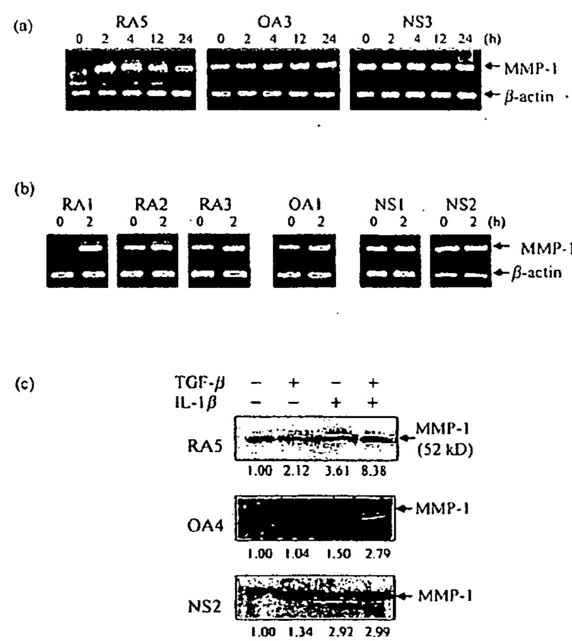

Fig. 4. Effect of TGF-β1 on MMP-1 expression. (a) Time-course analysis of MMP-1 mRNA expression was carried out by RT-PCR. (b) MMP-1 mRNA expression was analysed by RT-PCR in additional RA, OA and non-arthritic FLS cultures treated with TGF-β1 for 2 h (c) Protein expression of MMP-1 was analysed by immunoblotting. RA5 FLS were cultured in a low serum condition (0·2% FBS) for 24 h before stimulating with TGF-β1 and/or IL-1β. After 48 h, cell lysates were prepared and immunoblot analysis was performed using anti-MMP-1 antibody. The numbers below are the relative intensities of the bands measured by densitometry.

Activity of NF-κB and AP-1 in response to TGF-β1

Since NF-κB and AP-1 were transcription factors required for expression of many genes mediating inflammation, their activities were analysed after TGF-β1 treatment. TGF-β1 activated NF-κB and AP-1 in both RA and OA synoviocytes within 15 min and the activity of NF-κB and AP-1 lasted up to 60 min (Fig. 5a). Anti-p65 and anti-Fos antibodies caused supershift of the NF-κB and AP-1 bands, respectively (Fig. 5b). The specificity of DNA binding activity was also shown by the competition with unlabelled specific or irrelevant oligonucleotides.

DISCUSSION

TGF-β1 and its receptors are known to be expressed in RA synovial tissue. TGF-β exerts diverse and even opposite effects depending on the cell types and conditions. In the present study, we provided evidence that TGF-β1 could contribute to the inflammation and progression of the disease in RA and OA.

First, TGF-β1 increased mRNA expression of several inflammatory cytokines such as IL-1β, TNFα, IL-8 and MIP-1α in RA and OA FLS. This effect, interestingly, was specific to the arthritic FLS, since there was no or minimal increase in the mRNA expression of these cytokines in non-arthritic FLS. Protein expression of IL-1β and IL-8 was also enhanced by TGF-β1 in RA FLS. Moreover, TGF-β1 synergized with TNFα to increase expression of the IL-1β protein. This synergistic effect is potentially significant in *in vivo* situations, since both TNFα and TGF-β1 are present in the RA synovial tissue. mRNA expression of other members of CC chemokines such as MCP-1 and RANTES was not changed by TGF-β1 stimulation (data not shown).

Secondly, the level of MMP-1 expression was up-regulated by TGF-β1 in RA FLS. Synergistic effect of TGF-β1 and IL-1β in inducing MMP-1 protein expression was also observed. We also observed that MMP-3 mRNA expression was increased by TGF-β1 in RA FLS (data not shown). Thus, in RA, TGF-β1 appears to promote degradation of cartilages and the extracellular matrix proteins, rather than exerting an anabolic effect. Although TGF-β has been shown to reduce MMP-1 expression in many cases, up-regulation of MMP-1 by TGF-β1 is also reported [9,10,15,16]. In a ras-transformed HaCaT cell line, MMP-1 expression was stimulated by TGF-β and this effect was blocked by inhibitors of Erk1, 2 and p38 MAP kinases. Smad 3 and Smad 4 were shown to act together with c-Jun and c-Fos to activate the MMP-1 promoter.

Thirdly, TGF-β1 activated NF-κB and AP-1 in both RA and OA FLS. These transcription factors are required for the expression of many genes involved in the inflammatory process including IL-1β, TNFα and MMP-1 [17,18]. Therefore, TGF-β1 seems to increase the expression of inflammatory cytokines and MMP-1 through activation of these transcription factors. The significance of NF-κB and AP-1 in RA pathogenesis has been well documented [17–20]. Activation of these transcription factors was observed in RA synovial lining cells, which preceded arthritis development in collagen-induced arthritis (CIA).

Altogether, our results indicate that TGF-β1 can contribute for the inflammation and destruction of joints in RA and OA. The pro-inflammatory effects of TGF-β1 were specific to arthritic FLS. In non-arthritic FLS, TGF-β1 did not significantly induce expression of pro-inflammatory cytokines or MMP-1. It is well established that the effect of TGF-β is determined in the cellular context. The many different effects of TGF-β can be explained, in part, by interaction of the Smad complex in the nucleus with a set of partner proteins that are specific to a particular cell type under a particular condition [21]. These partners determine which genes the Smad complex will activate and how long this will last. Therefore, it is possible that a condition such as inflammation activates certain transcription factors that interact with Smad complex and influence the outcome of TGF-β stimulation.

Accumulating evidence suggests that RA FLS possesses unique transformed characteristics such as an anchorage-independent growth, lack of contact inhibition, elevated expression of proto-oncogenes and mutations in p53 [22,23]. However, these permanent changes do not seem to explain the differential response of RA FLS to TGF-β1. The inflammatory effect of TGF-β1 was also observed in OA FLS and while FLS cultures between the 4th and 10th passages gave similar responses to TGF-β1, the RA and OA FLS start to lose the proinflammatory responses to TGF-β1 after the 10th to 15th passages. Therefore, it is tempting to speculate that the inflammatory environment where the RA and OA FLS reside for a prolonged period might bring the proinflammatory effect of TGF-β1. An alternative explanation that cellular senescence affects the outcome of TGF-β stimulation cannot be ruled out. RA and OA FLS might be younger than the non-arthritic cells, since arthritic FLS can be maintained in culture much longer than the non-arthritic FLS.

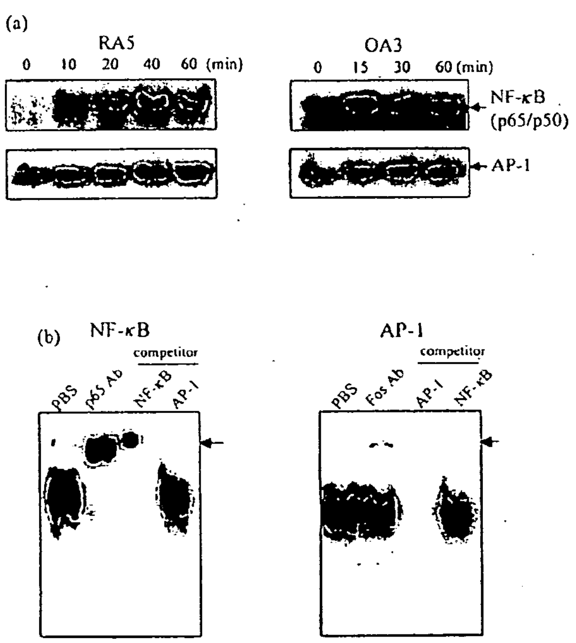

Fig. 5. Activation of NF-κB and AP-1 in response to TGF-β1. (a) After stimulation of RA and OA FLS with TGF-β1, nuclear extracts were prepared. EMSA was carried out as described in Materials and methods. (b) For supershift assay, nuclear extracts of RA5 were incubated with anti-p65 or anti-Fos antibodies for 30 min at 4°C before ^{32}P-labelled oligonucleotides were added. For cold competition, unlabelled NF-κB or AP-1 oligonucleotide was used in 100-fold molar excess.

ACKNOWLEDGEMENTS

We thank Dr Sangduk Kim for critical reading of the manuscript. This work was supported by grants from the Ministry of Health and Welfare, Republic of Korea (Good Health R&D Project, HMP-96-M-2-1035), the Brain Korea 21 Project in 2001 and the Medical Science Research Center at Korea University (1999-204).

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LEXSEE 853 F.2D 894

IN RE PATRICK H. O'FARRELL, BARRY A. POLISKY and DAVID H. GELFAND

No. 87-1486

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

853 F.2d 894; *1988 U.S. App. LEXIS 10951*; *7 U.S.P.Q.2D (BNA) 1673*

August 10, 1988, Decided

PRIOR HISTORY: [**1] Appealed from: U.S. Patent and Trademark Office Board of Patent Appeals and Interferences.

CASE SUMMARY:

PROCEDURAL POSTURE: Appellants sought review of the decision of U.S. Patent and Trademark Office Board of Patent Appeals and Interferences rejecting appellants' application under *35 U.S.C.S. § 103* because the claimed invention was obvious at the time the invention was made in view of a published paper by two of the coinventors.

OVERVIEW: Appellants alleged that at the time their article was published that there was significant unpredictability in the field of molecular biology so that the article would not have rendered the claimed method of translating heterologous DNA in bacteria obvious to one of ordinary skill in the art. In the alternative, appellants argued that the rejection was founded on the impermissible "obvious to try" standard. The court disagreed, holding that in light of the article, the claimed invention would have been obvious within the meaning of *35 U.S.C.S. § 103*. The article contained detailed enabling methodology for practicing the claimed invention, a suggestion to modify the prior art to practice the claimed invention, and evidence suggesting that it would be successful. Appellants foreclosed themselves from obtaining a patent because they published their pioneering studies more than a year before applying for a patent.

OUTCOME: The decision rejecting appellants' patent application was affirmed because the claimed invention was obvious in light of the published paper by two of the three co-inventors prior to filing their patent application.

LexisNexis(R) Headnotes

Civil Procedure > Trials > Judgment as Matter of Law > General Overview
Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN1] Obviousness under *35 U.S.C.S. § 103* is a question of law.

Evidence > Procedural Considerations > Objections & Offers of Proof > Timeliness
Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN2] An analysis of obviousness must be based on several factual inquiries: (1) the scope and content of the prior art; (2) the differences between the prior art and the claims at issue; (3) the level of ordinary skill in the art at the time the invention was made; and (4) objective evidence of nonobviousness, if any.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > Nonobviousness > Evidence & Procedure > General Overview
[HN3] Keeping the four statutory factors in mind and considering all of the evidence, the court must determine the correctness of the board's legal determination that the claimed invention as a whole would have been obvious to a person having ordinary skill in the art at the time the invention was made.

Patent Law > Nonobviousness > Elements & Tests > General Overview

[HN4] Obviousness does not require absolute predictability of success. Indeed, for many inventions that seem quite obvious, there is no absolute predictability of success until the invention is reduced to practice. There is always at least a possibility of unexpected results that would then provide an objective basis for showing that the invention, although apparently obvious, was in law nonobvious.

COUNSEL: J. Bruce McCubbrey, Fitch, Even, Tabin & Flannery, of San Francisco, California, argued for Appellant. Virginia H. Meyer, Fitch, Even, Tabin & Flannery, of San Francisco, California, was on the brief for Appellant.

Harris A. Pitlick, Associate Solicitor, of Arlington, Virginia, argued for Appellee. With him on the brief were Joseph F. Nakamura, Solicitor and Fred E. McKelvey, Deputy Solicitor.

JUDGES: Markey, Chief Judge, Rich and Nies, Circuit Judges.

OPINION BY: RICH

OPINION

[*895] RICH, Circuit Judge.

This appeal is from the decision of the United States Patent and Trademark Office Board of Patent Appeals and Interferences (board) affirming the patent examiner's final rejection of patent application Serial No. 180,424, entitled "Method and Hybrid Vector for Regulating Translation of Heterologous DNA in Bacteria." The application was rejected under *35 U.S.C. § 103* on the ground that the claimed invention would have been obvious at the time the invention was made in view of a published paper by two of the three coinventors, and a publication by Bahl, [**2] Marians & Wu, 1 *Gene* 81 (1976) (Bahl). We affirm.

The claimed invention is from the developing new field of genetic engineering. A broad claim on appeal reads:

> Claim 1. A method for producing a predetermined protein in a stable form in a transformed host species of bacteria comprising, providing a cloning vector which includes at least a substantial portion of a gene which is indigenous to the host species of bacteria and is functionally transcribed and translated in that species, said substantial portion of said indigenous gene further including the regulatory DNA sequences for RNA synthesis and protein synthesis but lacking the normal gene termination signal, and linking a natural or synthetic heterologous gene encoding said predetermined protein to said indigenous gene portion at its distal end, said heterologous gene being in proper orientation and having codons arranged in the same reading frame as the codons of said indigenous gene portion so that readthrough can occur from said indigenous gene portion into said heterologous gene in the same reading frame, said heterologous gene portion further containing sufficient DNA sequences to result in expression of a fused [**3] protein having sufficient size so as to confer stability on said predetermined protein when said vector is used to transform said host species of bacteria.

Illustrative embodiments are defined in more specific claims. For example:

> Claim 2. A method for producing a predetermined protein in a stable form in a transformed host species of bacteria, comprising, providing an *E. coli* plasmid having an operator, a promoter, a site for the initiation of translation, and at least a substantial portion of the beta-galactosidase gene of the *E. coli* lactose operon, said substantial portion of said beta-galactosidase gene being under the control of said operator, promoter and site for initiation of translation, said substantial portion of said beta-galactosidase gene lacking the normal gene termination signal, and linking a heterologous gene encoding said predetermined protein to said beta-galactosidase gene portion at its distal end, said heterologous gene being in proper orientation and having codons arranged in the same reading frame as the codons of the said beta-galactosidase gene portion so that readthrough can occur from said beta-galactosidase gene portion into said [**4] heterologous gene in the same reading frame, said heterologous gene portion further containing sufficient DNA sequences to result in expression of a fused protein having sufficient size so as to confer stability on said predetermined

protein when said vector is used to transform said host species of bacteria.

Claim 3. The method of Claim 2 wherein said *E. coli* plasmid comprises the plasmid designated pBGP120.

Although the terms in these claims would be familiar to those of ordinary skill in genetic engineering, they employ a bewildering vocabulary new to those who are not versed in molecular biology. An understanding of the science and technology on which these claims are based is essential before one can analyze and explain whether the claimed invention would have been obvious in light of the prior art.

I. *Background* [1]

1 Basic background information about molecular biology and genetic engineering, can be found in Alberts, Bray, Lewis, Raff, Roberts & Watson, *The Molecular Biology of the Cell*, 1-253, 385-481 (1983) [hereinafter *The Cell*]; Watson, Hopkins, Roberts, Steitz & Weiner, *The Molecular Biology of the Gene*, Vol. 1 (4th ed., 1987) 3-502 [hereinafter *The Gene*]. These standard textbooks were used to supplement the information in the glossary supplied by appellants. The description here is necessarily simplified and omits important facts and concepts that are not necessary for the analysis of this case.

[**5] Proteins are biological molecules of enormous importance. Proteins include enzymes [*896] that catalyze biochemical reactions, major structural materials of the animal body, and many hormones. Numerous patents and applications for patents in the field of biotechnology involve specific proteins or methods for making and using proteins. Many valuable proteins occur in nature only in minute quantities, or are difficult to purify from natural sources. Therefore, a goal of many biotechnology projects, including appellants' claimed invention, is to devise methods to synthesize useful quantities of specific proteins by controlling the mechanism by which living cells make proteins.

The basic organization of all proteins is the same. Proteins are large polymeric molecules consisting of chains of smaller building blocks, called *amino acids*, that are linked together covalently. [2] The chemical bonds linking amino acids together are called *peptide* bonds, so proteins are also called *polypeptides*. [3] It is the exact sequence in which the amino acids are strung together in a polypeptide chain that determines the identity of a protein and its chemical characteristics. [4] Although [**6] there are only 20 amino acids, they are strung together in different orders to produce the hundreds of thousands of proteins found in nature.

2 There are twenty amino acids: alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan, glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine, aspartic acid, glutamic acid, lysine, arginine, and histidine.
3 Proteins are often loosely called *peptides*, but technically proteins are only the larger peptides with chains of at least 50 amino acids, and more typically hundreds of amino acids. Some proteins consist of several polypeptide chains bound together covalently or noncovalently. The term "peptide" is broader than "protein" and also includes small chains of amino acids linked by peptide bonds, some as small as two amino acids. Certain small peptides have commercial or medical significance.
4 Polypeptide chains fold up into complex 3-dimensional shapes. It is the shape that actually determines many chemical properties of the protein. However, the configuration of a protein molecule is determined by its amino acid sequence. *The Cell* at 111-12; *The Gene* at 50-54.

[**7] To make a protein molecule, a cell needs information about the sequence in which the amino acids must be assembled. The cell uses a long polymeric molecule, DNA (deoxyriboneucleic acid), to store this information. The subunits of the DNA chain are called *nucleotides*. A nucleotide consists of a nitrogen-containing ring compound (called a *base*) linked to a 5-carbon sugar that has a phosphate group attached. [5] DNA is composed of only four nucleotides. They differ from each other in the base region of the molecule. The four bases of these subunits are adenine, guanine, cytosine, and thymine (abbreviated respectively as A, G, C and T). The sequence of these bases along the DNA molecule specifies which amino acids will be inserted in sequence into the polypeptide chain of a protein.

5 The sugar in DNA is deoxyribose, while the sugar in RNA, *infra*, is ribose. The sugar and phosphate groups are linked covalently to those of adjacent nucleotides to form the backbone of the long unbranched DNA molecule. The bases project from the chain, and serve as the "alphabet" of the genetic code.

DNA molecules actually consist of two chains tightly entwined as a double helix. The chains are not identical but instead are complementary: each A on one chain is paired with a T on the other chain, and each C has a correspond-

ing G. The chains are held together by noncovalent bonds between these complementary bases. This double helical structure plays an essential role in the replication of DNA and the transmission of genetic information. *See generally The Cell* at 98-106; *The Gene* at 65-79. However, the information of only one strand is used for directing protein synthesis, and it is not necessary to discuss the implication of the double-stranded structure of DNA here. RNA molecules, *infra*, are single stranded.

[**8] DNA molecules do not participate directly in the synthesis of proteins. DNA acts as a permanent "blueprint" of all of the [*897] genetic information in the cell, and exists mainly in extremely long strands (called *chromosomes*) containing information coding for the sequences of many proteins, most of which are not being synthesized at any particular moment. The region of DNA on the chromosome that codes for the sequence of a single polypeptide is called a *gene*. [6] In order to *express* a gene (the process whereby the information in a gene is used to synthesize new protein), a copy of the gene is first made as a molecule of RNA (ribonucleic acid).

6 Chromosomes also contain regions of DNA that are not part of genes, i.e., do not code for the sequence of amino acids in proteins. These include sections of DNA adjacent to genes that are involved in the control of transcription, *infra*, and regions of unknown function.

RNA is a molecule that closely resembles DNA. It differs, however, in that [**9] it contains a different sugar (ribose instead of deoxyribose) and the base thymine (T) of DNA is replaced in RNA by the structurally similar base, uracil (U). Making an RNA copy of DNA is called *transcription*. The transcribed RNA copy contains sequences of A, U, C, and G that carry the same information as the sequence of A, T, C, and G in the DNA. That RNA molecule, called *messenger RNA*, then moves to a location in the cell where proteins are synthesized.

The code whereby a sequence of nucleotides along an RNA molecule is translated into a sequence of amino acids in a protein (i.e., the "genetic code") is based on serially reading groups of three adjacent nucleotides. Each combination of three adjacent nucleotides, called a *codon*, specifies a particular amino acid. For example, the codon U-G-G in a messenger RNA molecule specifies that there will be a tryptophan molecule in the corresponding location in the corresponding polypeptide. The four bases A, G, C and U can be combined as triplets in 64 different ways, but there are only 20 amino acids to be coded. Thus, most amino acids are coded for by more than one codon. For example, both U-A-U and U-A-C code for tyrosine, [**10] and there are six different codons that code for leucine. There are also three codons that do not code for any amino acid (namely, U-A-A, U-G-A, and U-A-G). Like periods at the end of a sentence, these sequences signal the end of the polypeptide chain, and they are therefore called *stop codons*.

The cellular machinery involved in synthesizing proteins is quite complicated, and centers around large structures called *ribosomes* that bind to the messenger RNA. The ribosomes and associated molecules "read" the information in the messenger RNA molecule, literally shifting along the strand of RNA three nucleotides at a time, adding the amino acid specified by that codon to a growing polypeptide chain that is also attached to the ribosome. When a stop codon is reached, the polypeptide chain is complete and detaches from the ribosome.

The conversion of the information from a sequence of codons in an RNA molecule into the sequence of amino acids in a newly synthesized polypeptide is called *translation*. A messenger RNA molecule is typically reused to make many copies of the same protein. Synthesis of a protein is usually terminated by destroying the messenger RNA. (The information [**11] for making more of that protein remains stored in DNA in the chromosomes.)

The translation of messenger RNA begins at a specific sequence of nucleotides that bind the RNA to the ribosome and specify which is the first codon that is to be translated. Translation then proceeds by reading nucleotides, three at a time, until a stop codon is reached. If some error were to occur that shifts the frame in which the nucleotides are read by one or two nucleotides, all of the codons after this shift would be misread. For example, the sequence of codons [. . . *C-U-C-A-G-C-G-U-U-A-C-C-A*. . .] codes for the chain of amino acids [. .. leucine-serine-valine-threonine-. . .]. If the reading of these groups of three nucleotides is displaced by one nucleotide, such as [. . . *C-U-C-A-G-C-G-U-U-A-C-C-A* . . .], the resulting peptide chain would consist of [*898] [. . . serine-alanine-leucine-proline. . .]. This would be an entirely different peptide, and most probably an undesirable and useless one. Synthesis of a particular protein requires that the correct register or *reading frame* be maintained as the codons in the RNA are translated.

The function of messenger RNA is to carry [**12] genetic information (transcribed from DNA) to the protein synthetic machinery of a cell where its information is translated into the amino acid sequence of a protein. However, some kinds of RNA have other roles. For example, ribosomes contain several large strands of RNA that serve a structural function (*ribosomal RNA*). Chromosomes contain regions of DNA that code for the nu-

cleotide sequences of structural RNAs and these sequences are transcribed to manufacture those RNAs. The DNA sequences coding for structural RNAs are still called genes even though the nucleotide sequence of the structural RNA is never translated into protein.

Man, other animals, plants, protozoa, and yeast are *eucaryotic* (or eukaryotic) organisms: their DNA is packaged in chromosomes in a special compartment of the cell, the nucleus. Bacteria (*procaryotic* or prokaryotic organisms) have a different organization. Their DNA, usually a circular loop, is not contained in any specialized compartment. Despite the incredible differences between them, all organisms, whether eucaryote or procaryote, whether man or mouse or lowly bacterium, use the same molecular rules to make proteins under the control of genes. [**13] In all organisms, codons in DNA are transcribed into codons in RNA which is translated on ribosomes into polypeptides according to the same genetic code. Thus, if a gene from a man is transferred into a bacterium, the bacterium can manufacture the human protein. Since most commercially valuable proteins come from man or other eucaryotes while bacteria are essentially little biochemical factories that can be grown in huge quantities, one strategy for manufacturing a desired protein (for example, insulin) is to transfer the gene coding for the protein from the eucaryotic cell where the gene normally occurs into a bacterium.

Bacteria containing genes from a foreign source (*heterologous* genes) integrated into their own genetic makeup are said to be *transformed*. When transformed bacteria grow and divide, the inserted heterologous genes, like all the other genes that are normally present in the bacterium (*indigenous* genes), are replicated and passed on to succeeding generations. One can produce large quantities of transformed bacteria that contain transplanted heterologous genes. The process of making large quantities of identical copies of a gene (or other fragment of DNA) [**14] by introducing it into procaryotic cells and then growing those cells is called *cloning* the gene. After growing sufficient quantities of the transformed bacteria, the biotechnologist must induce the transformed bacteria to *express* the cloned gene and make useful quantities of the protein. This is the purpose of the claimed invention.

In order to make a selected protein by expressing its cloned gene in bacteria, several technical hurdles must be overcome. First the gene coding for the specific protein must be isolated for cloning. This is a formidable task, but recombinant DNA technology has armed the genetic engineer with a variety of techniques to accomplish it. [7] Next the isolated gene must be introduced into the host bacterium. This can be done by incorporating the gene into a cloning vector. A *cloning vector* is a piece of DNA that can be introduced into bacteria and will then replicate itself as the bacterial cells grow and divide. Bacteriophage (viruses that infect bacteria) can be used as cloning vectors, but plasmids were the type used by appellants. A *plasmid* is a small circular loop of DNA found in bacteria, separate from the chromosome, that replicates [**15] like a chromosome. It is like a tiny auxilliary chromosome containing only a few genes. Because of their small size, plasmids are convenient for the molecular biologist to isolate and work with. Recombinant DNA technology can be used to modify plasmids by splicing in cloned eucaryotic [*899] genes and other useful segments of DNA containing control sequences. Short pieces of DNA can even be designed to have desired nucleotide sequences, synthesized chemically, and spliced into the plasmid. One use of such chemically synthesized linkers is to insure that the inserted gene has the same reading frame as the rest of the plasmid; this is a teaching of the Bahl reference cited against appellants. A plasmid constructed by the molecular geneticist can be inserted into bacteria, where it replicates as the bacteria grow.

7 *See The Cell* at 185-194; *The Gene* at 208-10.

Even after a cloned heterologous gene has been successfully inserted into bacteria using a plasmid as a cloning vector, and replicates as [**16] the bacteria grow, there is no guarantee that the gene will be expressed, i.e., transcribed and translated into protein. A bacterium such as *E. coli* (the species of bacterium used by appellants) has genes for several thousand proteins. At any given moment many of those genes are not expressed at all. The genetic engineer needs a method to "turn on" the cloned gene and force it to be expressed. This is the problem appellants worked to solve.

II. *Prior art*

Appellants sought to control the expression of cloned heterologous genes inserted into bacteria. They reported the results of their early efforts in a publication, the three authors of which included two of the three coinventor-appellants (the Polisky reference [8]), that is undisputed prior art against them. Their strategy was to link the foreign gene to a highly regulated indigenous gene. Turning on expression of the indigenous gene by normal control mechanisms of the host would cause expression of the linked heterologous gene.

8 Polisky, Bishop & Gelfand, *A plasmid cloning vehicle allowing regulated expression of eukaryotic DNA in bacteria*, 73 Proc. Nat'l Acad. Sci. USA 3900 (1976).

[**17] As a controllable indigenous gene, the researchers chose a gene in the bacterium *E. coli* that makes beta-galactosidase. *Beta-galactosidase* is an en-

zyme needed to digest the sugar, lactose (milk sugar). When *E. coli* grows in a medium that contains no lactose, it does not make beta-galactosidase. If lactose is added to the medium, the gene coding for beta-galactosidase is expressed. The bacterial cell makes beta-galactosidase and is then able to use lactose as a food source. When lactose is no longer available, the cell again stops expressing the gene for beta-galactosidase.

The molecular mechanisms through which the presence of lactose turns on expression of the beta-galactosidase gene has been studied in detail, and is one of the best understood examples of how gene expression is regulated on the molecular level. The beta-galactosidase gene is controlled by segments of DNA adjacent to the gene. These *regulatory DNA sequences* (the general term used in Claim 1) include the *operator* and *promoter* sequences (specified in Claim 2). [9] The researchers constructed a plasmid containing the beta-galactosidase gene with its operator and promoter. This gene (with its [**18] regulatory sequences) was removed from the chromosome of *E. coli* where it is normally found and was transplanted to a plasmid that could be conveniently manipulated.

9 The *promoter* is a sequence of nucleotides where the enzyme that synthesizes RNA, *RNA polymerase*, attaches to the DNA to start the transcription of the beta-galactosidase gene. The *operator* is an overlapping DNA sequence that binds a small protein present in the cell, the lactose repressor protein. The lactose repressor protein binds to the operator and physically blocks the RNA polymerase from properly attaching to the promoter so that transcription cannot proceed. Lactose molecules interact with the lactose repressor protein and cause it to change its shape; after this change in shape it moves out of the way and no longer prevents the RNA polymerase from binding to the promoter. Messenger RNA coding for beta-galactosidase can then be transcribed. *See generally The Cell* at 438-39; *The Gene* at 474-80.

Restriction endonucleases [**19] are useful tools in genetic engineering. These enzymes cut strands of DNA, but only at places where a specific sequence of nucleotides is present. For example, one restriction endonuclease, called *EcoRI*, cuts DNA only at sites where the nucleotide sequence is [. . .-G-A-A-T-T-C-. . .]. With restriction [*900] enzymes the genetic engineer can cut a strand of DNA at very specific sites into just a few pieces. With the help of "repair" enzymes, other pieces of DNA can be spliced onto the cut ends. The investigators found that the plasmid which they had constructed contained only two sequences that were cut by EcoRI. They were able to eliminate one of these sites that was unwanted. They were then left with a plasmid containing the beta-galactosidase gene with its regulatory sequences, and a single EcoRI site that was within the beta-galactosidase gene and close to its stop codon. They named this plasmid that they had constructed pBGP120.

The next step was to cut the plasmid open at its EcoRI site and insert a heterologous gene from another organism. The particular heterologous gene they chose to splice in was a segment of DNA from a frog that coded for ribosomal RNA. The frog [**20] gene was chosen as a test gene for reasons of convenience and availability. The new plasmid created by inserting the frog gene was similar to pBGP120, but its beta-galactosidase gene was incomplete. Some codons including the stop codon were missing from its end, which instead continued on with the sequence of the frog ribosomal RNA gene. The investigators named this new plasmid pBGP123. They inserted this plasmid back into *E. coli* and grew sufficient quantities for study. They then fed the *E. coli* with lactose. As they had intended, the lactose turned on transcription of the beta-galactosidase gene in the plasmid. RNA polymerase moved along the plasmid producing a strange new kind of RNA: Each long strand of RNA first contained codons for the messenger RNA for beta-galactosidase and then continued without interruption with the codons for the frog ribosomal RNA. Thus, there was *readthrough* transcription in which the RNA polymerase first transcribed the indigenous (beta-galactosidase) gene and then "read through," i.e., continued into and through the adjacent heterologous (frog ribosomal RNA) gene. Although the RNA produced was a hybrid, it nevertheless contained a nucleotide [**21] sequence dictated by DNA from a frog. The researchers had achieved the first controlled transcription of an animal gene inside a bacterium.

The researchers had used a gene coding for a ribosomal RNA as their heterologous test gene. Ribosomal RNA is not normally translated into protein. Nevertheless, they were obviously interested in using their approach to make heterologous proteins in bacteria. They therefore examined the beta-galactosidase made by their transformed bacteria. Patrick O'Farrell, who was not a coauthor of the Polisky paper but was to become a coinventor in the patent application, joined as a collaborator. They found that beta-galactosidase from the transformed bacteria had a higher molecular weight than was normal. They concluded that the bacteria must have used their strange new hybrid RNA like any other messenger RNA and translated it into protein. When the machinery of protein synthesis reached the premature end of the sequence coding for beta-galactosidase it continued right on, three nucleotides at a time, adding whatever amino acid was coded for by those nucleotides, until a triplet

was reached with the sequence of a stop codon. The resulting polypeptide chains [**22] had more amino acids than normal beta-galactosidase, and thus a higher molecular weight. The researchers published their preliminary results in the Polisky article. They wrote:

> If the normal translational stop signals for [beta]-galactosidase are missing in pBGP120, in-phase translational readthrough into adjacent inserted sequences might occur, resulting in a significant increase in the size of the [beta]-galactosidase polypeptide subunit. In fact, we have recently observed that induced cultures of pBGP123 contain elevated levels of [beta]-galactosidase of higher subunit molecular weight than wild-type enzyme (P. O'Farrell, unpublished experiments). We believe this increase results from translation of *Xenopus* [frog] RNA sequences covalently linked to [messenger] RNA for [beta]-galactosidase, resulting in a fused polypeptide.

Polisky at 3904.

Since ribosomal RNA is never translated in normal cells, the polypeptide chain produced [*901] by translating that chain was not a naturally occuring, identified protein. The authors of the Polisky paper explicitly pointed out that if one were to insert a heterologous gene coding for a protein into their [**23] plasmid, it should produce a "fused protein" consisting of a polypeptide made of beta-galactosidase plus the protein coded for by the inserted gene, joined by a peptide bond into a single continuous polypeptide chain:

> It would be interesting to examine the expression of a normally translated eukaryotic sequence in pBGP120. If an inserted sequence contains a ribosome binding site that can be utilized in bacteria, production of high levels of a readthrough transcript might allow for extensive translation of a functional eukaryotic polypeptide. In the absence of an independent ribosome binding site, the eukaryotic sequence would be translated to yield a peptide covalently linked to [beta]-galactosidase. The extent of readthrough translation under *lac* control will depend on the number of translatable codons between the EcoRI site and the first in-phase nonsense [i.e., stop] codon in the inserted sequence.

Id.

III. *The Claimed Invention*

Referring back to Claims 1 through 3, it can be seen that virtually everything in the claims was present in the prior art Polisky article. The main difference is that in Polisky the heterologous gene was a gene [**24] for ribosomal RNA while the claimed invention substitutes a gene coding for a predetermined protein. Ribosomal RNA gene is not normally translated into protein, so expression of the heterologous gene was studied mainly in terms of transcription into RNA. Nevertheless, Polisky mentioned preliminary evidence that the transcript of the ribosomal RNA gene was translated into protein. Polisky further predicted that if a gene that codes for a protein were to be substituted for the ribosomal RNA gene, "a readthrough transcript might allow for extensive translation of a functional eukaryotic polypeptide." Thus, the prior art explicitly suggested the substitution that is the difference between the claimed invention and the prior art, and presented preliminary evidence suggesting that the method could be used to make proteins.

Appellants reduced their invention to practice some time in 1976 and reported their results in a paper that was published in 1978. [10] During 1977 they communicated their results to another group of researchers who used the readthrough translation approach to achieve the first synthesis of a human protein in bacteria. [11] Appellants filed an application to patent their [**25] invention on August 9, 1978, of which the application on appeal is a division.

10 O'Farrell, Polisky & Gelfand, *Regulated expression by readthrough translation from a plasmid-encoded beta-galactosidase*, 134 J. Bacteriol. 645 (1978). The heterologous genes expressed in these studies were not predetermined, but were instead unidentified genes of unknown origin. The authors speculated that they were probably genes from *E. coli* that were contaminants in the source of beta-galactosidase genes. *Id.* at 648.

11 Itakura, Hirose, Crea, Riggs, Heynecker, Bolivar & Boyer, *Expression in Escherichia coli of a chemically synthesized gene for the hormone somatostatin*, 198 Science 1056 (1977). A pioneering accomplishment of the Itakura group is that the gene was not from a human source, but instead was entirely synthesized in the laboratory using chemical methods. It is not clear whether the appellants communicated only the results reported in the Polisky publication or whether they communicated the complete claimed invention.

[**26] IV. *The Obviousness Rejection*

The application was rejected under *35 U.S.C. § 103*. The position of the examiner and the Board is, simply, that so much of the appellant's method was revealed in the Polisky reference that making a protein by substituting its gene for the ribosomal RNA gene in Polisky (as suggested by Polisky) would have been obvious to one of ordinary skill in the art at the time that the invention was made.

The claims specify that the heterologous gene should be inserted into the plasmid in the same orientation and with the same reading frame as the preceding portion of [*902] the indigenous gene. In view of this limitation, the *§ 103* rejection was based either on Polisky alone (supplemented by the fact that the importance of orientation and reading frame was well known in the prior art) or in combination with the Bahl reference which describes a general method for inserting a piece of chemically synthesized DNA into a plasmid. Bahl teaches that this technique could be used to shift the sequence of DNA inserted into a plasmid into the proper [**27] reading frame.

Appellants argue that at the time the Polisky article was published, there was significant unpredictability in the field of molecular biology so that the Polisky article would not have rendered the claimed method obvious to one of ordinary skill in the art. Even though there was speculation in the article that genes coding for proteins could be substituted for the ribosomal RNA gene and would be expressed as readthrough translation into the protein, this had never been done. Appellants say that it was not yet certain whether a heterologous protein could actually be produced in bacteria, and if it could, whether additional mechanisms or methods would be required. They contend that without such certainty the predictions in the Polisky paper, which hindsight now shows to have been correct, were merely invitations to those skilled in the art to try to make the claimed invention. They argue that the rejection amounts to the application of a standard of "obvious to try" to the field of molecular biology, a standard which this court and its predecessors have repeatedly rejected as improper grounds for a *§ 103* rejection. *E.g., In re Fine, 837 F.2d 1071, 1075, 5 USPQ2d 1596, 1599 (Fed. Cir. 1988)*; [**28] *In re Geiger, 815 F.2d 686, 688, 2 USPQ2d 1276, 1278 (Fed. Cir. 1987)*; *In re Merck & Co., Inc., 800 F.2d 1091, 1097, 231 USPQ 375, 379 (Fed. Cir. 1986)*; *In re Antonie, 559 F.2d 618, 620, 195 USPQ 6, 8 (CCPA 1977)*.

[HN1] Obviousness under *§ 103* is a question of law. *Panduit Corp. v. Dennison Mfg. Co., 810 F.2d 1561, 1568, 1 USPQ2d 1593, 1597* (Fed. Cir.), *cert. denied, 481 U.S. 1052, 107 S. Ct. 2187, 95 L. Ed. 2d 843 (1987)*. [HN2] An analysis of obviousness must be based on several factual inquiries: (1) the scope and content of the prior art; (2) the differences between the prior art and the claims at issue; (3) the level of ordinary skill in the art at the time the invention was made; and (4) objective evidence of nonobviousness, if any. *Graham v. John Deere Co., 383 U.S. 1, 17-18, 148 USPQ 459, 467, 15 L. Ed. 2d 545, 86 S. Ct. 684 (1966)*. *See, e.g., Custom Accessories, Inc. v. Jeffrey-Allan Indus., 807 F.2d 955, 958, 1 USPQ2d 1196, 1197 (Fed. Cir. 1986)*. [**29] The scope and content of the prior art and the differences between the prior art and the claimed invention have been examined in sections II and III, *supra*. Appellants say that in 1976 those of ordinary skill in the arts of molecular biology and recombinant DNA technology were research scientists who had "extraordinary skill in relevant arts" and "were among the brightest biologists in the world." Objective evidence of nonobviousness was not argued.

[HN3] With the statutory factors as expounded by *Graham* in mind and considering all of the evidence, this court must determine the correctness of the board's legal determination that the claimed invention as a whole would have been obvious to a person having ordinary skill in the art at the time the invention was made. We agree with the board that appellant's claimed invention would have been obvious in light of the Polisky reference alone or in combination with Bahl within the meaning of *§ 103*. Polisky contained detailed enabling methodology for practicing the claimed invention, a suggestion to modify the prior art to practice the claimed [**30] invention, and evidence suggesting that it would be successful.

Appellants argue that after the publication of Polisky, successful synthesis of protein was still uncertain. They belittle the predictive value of the observation that expression of the transcribed RNA in Polisky produced beta-galactosidase with a greater than normal molecular weight, arguing that since ribosomal RNA is not normally translated, the polypeptide chains that were added to the end of the beta-galactosidase [*903] were "junk" or "nonsense" proteins. This characterization ignores the clear implications of the reported observations. The Polisky study directly proved that a readthrough transcript messenger RNA had been produced. The preliminary observation showed that this messenger RNA was read and used for successful translation. It was well known in the art that ribosomal RNA was made of the same nucleotides as messenger RNA, that any sequence of nucleotides could be read in groups of three as codons, and that reading these codons should specify a polypeptide chain that would elongate until a stop codon was encountered. The preliminary observations thus showed that codons beyond the end of the beta-galactosidase [**31] gene were being translated into peptide chains.

This would reasonably suggest to one skilled in the art that if the codons inserted beyond the end of the beta-galactosidase gene coded for a "predetermined protein," that protein would be produced. In other words, it would have been obvious and reasonable to conclude from the observation reported in Polisky that since nonsense RNA produced nonsense polypeptides, if meaningful RNA was inserted instead of ribosomal RNA, useful protein would be the result. The relative shortness of the added chains is also not a source of uncertainty, since one skilled in the art would have known that a random sequence of nucleotides would produce a stop codon before the chain got too long. [12]

12 The patent application indicates that chains as long as 60 amino acids were added, which is hardly a trivial length of polypeptide.

Appellants complain that since predetermined proteins had not yet been produced in transformed bacteria, there was uncertainty as to whether this could [**32] be done, and that the rejection is thus founded on an impermissible "obvious to try" standard. It is true that this court and its predecessors have repeatedly emphasized that "obvious to try" is not the standard under *§ 103*. However, the meaning of this maxim is sometimes lost. Any invention that would in fact have been obvious under *§ 103* would also have been, in a sense, obvious to try. The question is: when is an invention that was obvious to try nevertheless nonobvious?

The admonition that "obvious to try" is not the standard under *§ 103* has been directed mainly at two kinds of error. In some cases, what would have been "obvious to try" would have been to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful. *E.g., In re Geiger, 815 F.2d at 688, 2 USPQ2d at 1278; Novo Industri A/S v. Travenol Laboratories, Inc., 677 F.2d 1202, 1208, 215 USPQ 412, 417 (7th Cir. 1982); In re Yates, 663 F.2d 1054, 1057, 211 USPQ 1149, 1151 (CCPA 1981);* [**33] *In re Antonie, 559 F.2d at 621, 195 USPQ at 8-9*. In others, what was "obvious to try" was to explore a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it. *In re Dow Chemical Co., 837 F.2d 469, 473, 5 USPQ2d 1529, 1532 (Fed. Cir. 1988); Hybritech, Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1380, 231 USPQ 81, 90-91 (Fed. Cir. 1986), cert. denied, 480 U.S. 947, 107 S. Ct. 1606, 94 L. Ed. 2d 792 (1987); In re Tomlinson, 53 C.C.P.A. 1421, 363 F.2d 928, 931, 150 USPQ 623, 626 (CCPA 1966)*. Neither of these situations applies here.

[HN4] Obviousness does not require absolute predictability of success. Indeed, for many inventions that seem quite obvious, there is no absolute predictability of success until the invention is reduced to practice. There is always at least a possibility of unexpected results, that would then provide an objective basis for [**34] showing that the invention, although apparently obvious, was in law nonobvious. *In re Merck & Co., 800 F.2d at 1098, 231 USPQ at 380; Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co., 730 F.2d 1452, 1461,* [*904] *221 USPQ 481, 488 (Fed. Cir. 1984); In re Papesch, 50 C.C.P.A. 1084, 315 F.2d 381, 386-87, 137 USPQ 43, 47-48 (CCPA 1963)*. For obviousness under *§ 103*, all that is required is a reasonable expectation of success. *In re Longi, 759 F.2d 887, 897, 225 USPQ 645, 651-52 (Fed. Cir. 1985); In re Clinton, 527 F.2d 1226, 1228, 188 USPQ 365, 367 (CCPA 1976)*. The information in the Polisky reference, when combined with the Bahl reference provided such a reasonable expectation of success.

Appellants published their pioneering studies of the expression of frog ribosomal RNA genes in bacteria more than a year before they applied for a patent. After providing virtually all of their method to the public without applying for a patent within a year, they foreclosed themselves from obtaining a patent on a method that would have been obvious from their publication to those of ordinary [**35] skill in the art, with or without the disclosures of other prior art. The decision of the board is

AFFIRMED.

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Reference No. 10

LEXSEE 491 F.3D 1342

PHARMASTEM THERAPEUTICS, INC., Plaintiff-Appellant, v. VIACELL, INC., Defendant-Cross Appellant, and CRYO-CELL INTERNATIONAL, INC., COR-CELL, INC., Defendants-Cross Appellants, and CBR SYSTEMS, INC. (formerly known as Cord Blood Registry, Inc.), Defendant-Cross Appellant, and BIRTH-CELLS TECHNOLOGY, INC. and BIO-CELL, INC., Defendants.

05-1490, 05-1551

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

491 F.3d 1342; 2007 U.S. App. LEXIS 16245; 83 U.S.P.Q.2D (BNA) 1289

July 9, 2007, Decided

SUBSEQUENT HISTORY: US Supreme Court certiorari denied by *Pharmastem Therapeutics v. Viacell, 128 S. Ct. 1655, 170 L. Ed. 2d 355, 2008 U.S. LEXIS 2477 (U.S., Mar. 17, 2008)*

PRIOR HISTORY: [**1]
Appealed from: United States District Court for the District of Delaware, Chief Judge Gregory M. Sleet.
Pharmastem Therapeutics v. Viacell, Inc., 2004 U.S. Dist. LEXIS 25176 (D. Del., Dec. 14, 2004)
Pharmastem Therapeutics, Inc. v. ViaCell Inc., 2004 U.S. Dist. LEXIS 18638 (D. Del., Sept. 15, 2004)

DISPOSITION: AFFIRMED IN PART, REVERSED IN PART, and REMANDED.

CASE SUMMARY:

PROCEDURAL POSTURE: Appellant owner of patents sued cross-appellant competitors, alleging that the competitors infringed the patents relating to a medical procedure for treating persons with compromised blood and immune systems. The owner and the competitors cross-appealed the orders of the U.S. District Court for the District of Delaware which granted judgment of non-infringement as a matter of law and denied judgment as a matter of law with regard to obviousness.

OVERVIEW: The patents recited a process for collecting umbilical cord blood, testing the blood for suitability for later use, cryopreserving the blood, and infusing the blood into an individual whose hematopoietic stem cells were destroyed to reconstitute the recipient's blood and immune system. The competitors offered a service to families for collection and cryopreservation of cord blood for possible later use. The appellate court first held that the patent relating to the composition of stem cells was not infringed, since the general evidence, which did not include direct testing, failed to establish that the cord blood preserved by the competitors contained enough stem cells to reconstitute a human adult, as required by the patent. Further, the competitors did not contributorily infringe the patent relating to the reconstitution method, since the competitors only sold the service of collecting and preserving the stem cells while transplant physicians completed the patented method by thawing and infusing the stem cells. Also, the patents were invalid for obviousness since the inventors merely proved the existence of stem cells in cord blood which was inferred by prior art.

OUTCOME: The order granting judgment of non-infringement was affirmed, the order denying judgment of invalidity based on obviousness was reversed, and the case was remanded for entry of judgment of patent invalidity.

LexisNexis(R) Headnotes

Patent Law > Infringement Actions > General Overview
[HN1] There is no prohibition against using the admissions of a party, whether in the form of marketing materials or otherwise, as evidence in an patent infringement action; such admissions are entitled to weight along with all other evidence of infringement.

Patent Law > Infringement Actions > Infringing Acts > Contributory, Indirect & Induced Infringement
[HN2] *35 U.S.C.S. § 271(c)* provides that whoever offers to sell or sells a component of a patented machine, manufacture, combination or composition, or a material or apparatus for use in practicing a patented process, constituting a material part of the invention, knowing the same to be especially made or especially adapted for use in an infringement of such patent is liable as a contributory infringer.

Contracts Law > Types of Contracts > Bailments
[HN3] The recognized distinction between bailment and sale is that, when the identical article is to be returned in the same or in some altered form, the contract is one of bailment, and the title to the property is not changed. On the other hand, when there is no obligation to return the specific article, and the receiver is at liberty to return another thing of value, he becomes a debtor to make the return, and the title to the property is changed.

Patent Law > Infringement Actions > Infringing Acts > Contributory, Indirect & Induced Infringement
[HN4] *35 U.S.C.S. § 271(c)* provides, in part, that a contributory infringer is one who offers to sell or sells within the United States a patented machine, manufacture, combination or composition, or a material or apparatus for use in practicing a patented process. Although that language describes in various different ways the items that may be sold for purposes of creating liability for contributory infringement, all of the descriptions refer to the sale of a product of some sort; none of them refer to the provision of a service. Under the plain language of the statute, a person who provides a service that assists another in committing patent infringement may be subject to liability under *§ 271(b)* for active inducement of infringement, but not under *§ 271(c)* for contributory infringement.

Patent Law > Infringement Actions > Defenses > Patent Invalidity > Fact & Law Issues
Patent Law > Jurisdiction & Review > Standards of Review > De Novo Review
Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN5] Obviousness with regard to validity of a patent is a legal conclusion that an appellate court reviews de novo. A statutory standard requires the appellate court to decide whether the subject matter of the claimed invention would have been obvious at the time the invention was made to a person of ordinary skill in the art to which the subject matter of the invention pertains. *35 U.S.C.S. § 103(a)*. Underpinning that legal issue are factual questions relating to the scope and content of the prior art, the differences between the prior art and the claimed invention, the level of ordinary skill in the art, and any relevant secondary considerations, such as commercial success, long-felt need, and the failure of others.

Civil Procedure > Trials > Judgment as Matter of Law > General Overview
Civil Procedure > Appeals > Standards of Review > De Novo Review
[HN6] Under Third Circuit law, which dictates the standard for reviewing the denial of the motion for judgment as a matter of law (JMOL), an appellate court reviews a district court's action de novo by reapplying the JMOL standard applied by the district court.

Civil Procedure > Trials > Judgment as Matter of Law > Postverdict Judgments
Patent Law > Jurisdiction & Review > Standards of Review > Substantial Evidence
Patent Law > Nonobviousness > General Overview
[HN7] In reviewing a denial of a motion for judgment as a matter of law on the issue of obviousness in a patent case, an appellate court examines the evidence in the light most favorable to the verdict and determines whether a reasonable jury could have found all the facts necessary to support the verdict of nonobviousness, i.e., whether substantial evidence supports the verdict.

Evidence > Procedural Considerations > Burdens of Proof > Allocation
Evidence > Procedural Considerations > Burdens of Proof > Clear & Convincing Proof
Patent Law > Infringement Actions > Defenses > Patent Invalidity > Grounds
Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN8] In a case alleging patent invalidity based on obviousness, the burden falls on a patent challenger to show by clear and convincing evidence that a person of ordinary skill in the art would have had reason to attempt to make the composition or device, or carry out the claimed process, and would have had a reasonable expectation of success in doing so.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN9] Admissions in a patent specification regarding prior art are binding on the patentee for purposes of a later inquiry into obviousness.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN10] Scientific confirmation of what is already believed to be true may be a valuable contribution, but it does not give rise to a patentable invention.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN11] Obviousness does not require absolute predictability of success. Indeed, for many inventions that seem quite obvious, there is no absolute predictability of success until the invention is reduced to practice. On the other hand, an invention would not be invalid for obviousness if the inventor would have been motivated to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful. Likewise, an invention would not be deemed obvious if all that was suggested was to explore a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it.

Evidence > Procedural Considerations > Burdens of Proof > Allocation
Patent Law > Infringement Actions > Defenses > Patent Invalidity > Validity Presumption
Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN12] When a party asserting patent invalidity relies on references that were considered during examination or reexamination, that party bears the added burden of overcoming the deference that is due to a qualified government agency presumed to have done its job.

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JUDGES: Before NEWMAN, BRYSON, and PROST, Circuit Judges. Opinion for the court filed by Circuit [**2] Judge BRYSON. Dissenting opinion filed by Circuit Judge NEWMAN.

OPINION BY: BRYSON

OPINION

[*1346] BRYSON, Circuit Judge.

This patent infringement action was brought by appellant PharmaStem Therapeutics, Inc., in the United States District Court for the District of Delaware. PharmaStem sued six defendants (four of which are appellees before us in this appeal), alleging that the defendants had infringed two patents owned by PharmaStem, *U.S. Patent No. B1 5,004,681* ("the *'681 patent*") and U.S. *Patent No. 5,192,553* ("the *'553 patent*"), a continuation-in-part of the *'681 patent.* At the conclusion of the trial, the jury returned verdicts for PharmaStem on both patents, finding both patents infringed and not invalid. The jury also rejected the defendants' counterclaims of inequitable conduct and violation of the antitrust laws.

The defendants filed motions for judgment as a matter of law ("JMOL") and a new trial. In response, the district court initially entered an order granting a new trial on the issue of infringement of the *'681 patent* and JMOL of noninfringement [*1347] as to the *'553 patent*. Subsequently, however, the court vacated the new trial order as to the *'681 patent* and instead entered JMOL of noninfringement as to [**3] that patent. The court denied the defendants' JMOL motions with respect to various asserted grounds of patent invalidity. PharmaStem now appeals from the JMOL orders on infringement, and the defendants cross-appeal from the court's refusal to grant JMOL on invalidity. We affirm the district court's judgment as to the infringement issues. With respect to the counterclaim of invalidity for obviousness, however, we reverse the judgment and direct the entry of judgment for the defendants.

I

The two patents in suit recite compositions and methods relating to a medical procedure for treating per-

sons with compromised blood and immune systems. The treatment is based on the discovery that blood from a newborn infant's umbilical cord is a rich source of a type of stem cells useful for rebuilding an individual's blood and immune system after that system has been compromised by disease or a medical treatment such as chemotherapy.

Stem cells are fundamental (or "immature," or "primitive") cells from which specialized (or "mature") cells derive. Hematopoietic stem cells are stem cells that are ultimately responsible for producing the various specialized cells of the blood and immune (or "hematopoietic") [**4] system. Hematopoietic stem cells produce progenitor cells and more hematopoietic stem cells. The progenitor cells, which are less primitive than the stem cells, in turn give rise to the variety of specialized cells that constitute the blood and immune system.

Although hematopoietic stem cells are present in various types of human tissue, they are found in unusually high concentration and potency in umbilical cord blood. The *'681* and *'553 patents* describe a process for collecting a newborn infant's umbilical cord blood at the time of birth, testing it for suitability for later use, preserving it through cryopreservation, and infusing it into an individual (either the donor or another person, preferably one with a closely matched blood type) whose hematopoietic stem cells have been destroyed. The object of such transplantations is to effect grafting. A successful graft results when the donor's stem cells migrate into the recipient's bone marrow, resulting in the renewed production of normal, specialized blood cells and ultimately the reconstitution of the recipient's entire blood and immune system.

As issued, the *'681 patent* contained very broad claims. Claim 1 recited a composition comprising [**5] "a plurality of viable human neonatal or fetal hematopoietic stem cells derived from the blood [and a] cryopreservative." In reexamination, several of the original claims were cancelled. Claim 1 was amended to read as follows:

> A cryopreserved therapeutic composition comprising viable human neonatal or fetal hematopoietic stem cells derived from the umbilical cord blood or placental blood of a single human collected at the birth of said human, in which said cells are present in an amount sufficient to effect hematopoietic reconstitution of a human adult; and an amount of cryopreservative sufficient for cryopreservation of said cells.

Claim 2, which is dependent on claim 1, was amended to recite the composition of claim 1 "which further comprises viable human neonatal or fetal hematopoietic progenitor cells."

Each of the defendants offers a service to families of newborn infants in which blood from the infant's umbilical cord is collected and cryopreserved for possible later use. The defendants represent in their promotional literature that the preserved [*1348] cord blood may be useful for reconstituting the donor's hematopoietic system in the event that system is damaged or destroyed as a result [**6] of disease or other causes. Some of the promotional literature advises that the preserved cord blood may also be useful for treating closely related members of the infant's family.

In the infringement action brought against all six defendants, PharmaStem asserted claims 1 and 2 of the *'681 patent*, as amended in reexamination, and claims 13, 19, 47, 53, and 57 of the *'553 patent*. Claims 13, 47, and 57 of the *'553 patent* are independent claims. Claim 13 provides as follows:

> A method for hematopoietic or immune reconstitution of a human comprising:
>
> (a) isolating human neonatal or fetal blood components containing hematopoietic stem cells;
>
> (b) cryopreserving the blood components;
>
> (c) thawing the blood components; and
>
> (d) introducing the blood components into a suitable human host, such that the hematopoietic stem cells are viable and can proliferate with the host.

Claim 47 is similar except that it refers to the blood components "containing hematopoietic stem and progenitor cells." Dependent claims 19 and 53 add that the blood components are isolated by collection from an umbilical cord. Independent claim 57 provides as follows:

> A method for hematopoietic or immune reconstitution of a human comprising [**7] introducing into the human a composition comprising human neonatal or fetal hematopoietic stem cells derived from the blood, in which the stem cells have been previously cryopreserved.

II

Following the jury's verdict finding infringement of both patents by all four appellants, the district court granted the defendants' JMOL motions and entered a judgment of noninfringement with respect to both patents. The court agreed with the defendants that, in light of the legal theories pressed by PharmaStem at trial, the evidence failed to show that any of the defendants had infringed any of the asserted claims of either patent in suit.

As to infringement of the asserted *'681 patent* claims, the district court focused on the requirement that the recited compositions contain stem cells "in an amount sufficient to effect hematopoietic reconstitution of a human adult." To prove infringement, the court explained, PharmaStem was required to adduce evidence that the defendants' cord blood units contained a sufficient supply of stem cells to effect successful reconstitution of an adult. The court concluded that PharmaStem had failed to do so.

In addressing the sufficiency of the evidence on that issue, the [**8] trial court first ruled that it should have excluded the trial testimony of Dr. Mary Hendrix, PharmaStem's expert witness on infringement. The court noted that although Dr. Hendrix was "an accomplished stem cell biologist," she based her infringement opinion "entirely on an analysis of the defendants' marketing materials, without ever considering any data regarding the composition of the defendants' cord blood units." The court explained that Dr. Hendrix was not qualified as an expert in marketing or advertising and, in any event, "her so-called analysis of the defendants' marketing materials was well within the jury's common knowledge, common sense and common experience." The court pointed out that Dr. Hendrix's opinion that all of the defendants' cord blood units infringe [*1349] was based on her conclusion that the defendants' promotional materials "promise stem cells for pediatric and adult transplantation." In that respect, according to the court, "her opinion of infringement is no more than a lay-person's interpretation of the defendants' marketing materials." The court therefore ruled that her testimony should have been excluded and that "permitting PharmaStem to couch its presentation [**9] of this evidence in the form of an expert opinion was an error."

The district court then pointed out that the evidence at trial overwhelmingly indicated that not all units of cord blood obtained from a single individual at birth contain enough stem cells to reconstitute an adult. The court explained that PharmaStem did not attempt to prove by testing or by reference to data collected by the defendants that at least some of the cord blood samples preserved by the defendants satisfied that requirement. Instead, the court noted, PharmaStem adopted the strategy of trying to prove, principally through representations made by the defendants in their marketing materials and other documents, that all of the preserved cord blood samples infringed. As a consequence, the court explained, PharmaStem "presented no evidence to the jury from which it could conclude that any specific cord blood unit or units stored by any of the defendants contained stem cells in a sufficient amount to reconstitute a human adult." Because there was "no legally sufficient evidentiary basis for a reasonable jury to find that all, or any specific number, of the defendants' cord blood units infringe the *'681 patent*," the [**10] court granted the defendants' motion for JMOL as to the *'681 patent* claims.

As to infringement of the *'553 patent*, the district court granted the defendants' motions for JMOL because it concluded that PharmaStem had failed to prove that the defendants were guilty of contributory infringement, which was PharmaStem's theory of liability. Under the court's instructions, the jury was required to answer three questions in the affirmative in order to find that any of the defendants contributorily infringed the *'553 patent*. Specifically, the jury was required to find (1) that cryopreserved cord blood has no substantial noninfringing uses; (2) that the defendants and transplant physicians were acting in concert or working together to complete the process of infringement of the asserted claims of the *'553 patent*; and (3) that the defendants contributorily infringed "by selling or offering to sell cryopreserved cord blood that was actually used by a third party in the direct infringement" of the asserted claims. The court held that there was sufficient evidence at trial to support the jury's affirmative answers to the first two questions. With respect to the third question, however, the court [**11] held that there was no evidence in the record to support the jury's affirmative answer. The court explained its ruling as follows:

> It is undisputed that the defendants do not own the cord blood units. Rather the units are owned by the clients, or families, and the defendants in turn provide services with respect to the processing and storing of the compositions. Although the defendants charge enrollment, processing, and banking fees with respect to their storage services, they do not sell or offer to sell the cord blood units. Indeed, the record evidence on this issue is clear that the defendants sell a service, not cord blood units.

Because the court ruled that liability for contributory infringement "is clearly dependent upon the accused infringer's selling or offering to sell a component of the patented process, here cord blood units," the court held that the jury's verdict could not stand. The court therefore granted [*1350] JMOL as to the asserted claims of the *'553 patent*.

Although granting the defendants' motions for JMOL as to infringement, the district court denied their motions for JMOL of invalidity with respect to the asserted claims. As to obviousness, the court ruled that the evidence [**12] at trial showed that there were problems associated with the use of other transplant tissues, such as bone marrow and adult blood, and that there was "tremendous skepticism in the transplant field regarding the use of cord blood as a transplant tissue." Although the court stated that a jury could have found from the evidence that the asserted claims would have been obvious, the court ruled that the evidence was sufficient to entitle the jury to conclude that "prior to the inventions of the Patents-In-Suit, those in the field of hematopoietic reconstitution would not have expected cord blood to be a successful transplant tissue."

As to anticipation, the district court again ruled that the evidence was sufficient to support the jury's verdict that the prior art reference on which the defendants relied did not anticipate the asserted claims. The court explained that the jury was entitled to find that the prior art reference did not prove that there were stem cells in umbilical cord blood, and that the jury could reasonably have concluded that the suggestion of introducing stem cells into a human host was not "a sufficiently enabling disclosure to warrant a finding of anticipation."

Finally, [**13] the district court rejected the defendants' argument that the *'681 patent* was invalid for indefiniteness. The court acknowledged that claim 1 of the *'681 patent* does not specify a particular number of cells or volume of blood that is required to infringe. Nonetheless, the court concluded that "the record supports that the *'681* Patent's claim language is as precise as the subject matter permits." Moreover, the court ruled that the record contained evidence establishing that "a person of skill in the art would have understood what an amount of cord blood stem cells sufficient to effect hematopoietic reconstitution of a human adult means."

PharmaStem has appealed from the portion of the district court's judgment granting JMOL of noninfringement with respect to both patents. The defendants have cross-appealed from the portion of the judgment upholding the jury's verdict that the two patents are not invalid on grounds of anticipation, obviousness, or (in the case of the *'681 patent*) indefiniteness.

III

With respect to infringement of the *'681 patent*, the dispute on appeal is a narrow one. The only contested limitation of the asserted claims is the limitation requiring that the claimed composition [**14] contain neonatal or fetal hematopoietic stem cells "in an amount sufficient to effect hematopoietic reconstitution of a human adult." PharmaStem contends that all of the cord blood samples the defendants have preserved infringe claim 1 of the *'681 patent* because the evidence at trial was sufficient to show that all those cord blood units contained enough stem cells to effect the hematopoietic reconstitution of a human adult. The defendants contend that PharmaStem failed to prove that any of their cryopreserved samples satisfy that limitation.

As the district court noted, PharmaStem did not attempt to use direct testing or other scientific evidence to prove that any particular cord blood sample or group of samples preserved by any of the defendants contained enough stem cells to reconstitute a human adult. Instead, PharmaStem relied on indirect evidence in the form of advertising and other materials generated by the defendants, scientific evidence [*1351] relating to stem cell research in general, testimony from representatives of the defendants, and testimony by their own expert witness, Dr. Hendrix. The district court, however, concluded that PharmaStem's evidence did not constitute substantial [**15] evidence in support of PharmaStem's theory of infringement.

A

The trial court was correct in ruling that the evidence of the defendants' advertising and other materials did not provide a sufficient basis for a finding of infringement. That evidence consisted of various statements by each of the defendants that the cord blood samples they preserved could be potentially useful not only for the donor but also for the donor's relatives, including adult relatives.

To be sure, [HN1] there is no prohibition against using the admissions of a party, whether in the form of marketing materials or otherwise, as evidence in an infringement action; such admissions are entitled to weight along with all other evidence of infringement. In this case, however, while the defendants' statements touted the possible therapeutic uses the cord blood might have for the child and members of the child's family in the future, none of the statements represented that the stem cells in any of the cryopreserved cord blood samples were sufficient in number to effect hematopoietic reconstitution of an adult, as is required by claim 1 of the reexamined *'681 patent*. Instead, the defendants' statements emphasized the potential [**16] therapeutic usefulness of the cord blood in general and referred to future uses of stored blood in adult transplants only as possibilities.

For example, PharmaStem introduced a statement from a website maintained by defendant CBR Systems, Inc., which referred to the number and character of cord blood transplants worldwide as of that time. The statement recited that "umbilical cord blood has been used in more than 2,500 transplants by children and adults. In many cases, the cord blood was used by the baby's sibling. Other transplants have occurred for the newborn himself, the newborn's mother, father, and the newborn's cousin." With respect to its own preserved cord blood units, CBR stated that it had provided "over two dozen samples for use in transplantation," that most have been used for siblings, but that in one instance the newborn's "cord blood stem cells were transplanted to her mother to treat chronic myelogenous leukemia."

Those statements fall short of proving that any (much less all) of CBR's cord blood samples contained enough stem cells to reconstitute an adult. The first statement simply recited that among the 2500 worldwide transplants, some had been conducted on adults. [**17] The second statement reflected that one such adult transfer was attempted with a CBR cord blood sample. Neither statement made any representation whether or to what extent the particular transplants had succeeded in reconstituting the adults' hematopoietic systems. Nor did the specific reference to the one adult transplant represent that the transplant was successful or that only a single unit of cord blood was used in the transplant. Those gaps in the proof are significant, because the evidence showed that as of the time of trial the great majority of all cord blood transplants worldwide had been for the treatment of children. In addition, the evidence showed that in most cases involving adult transplantations, the transplant physicians had used two units of cord blood, not the one unit obtained at the time of a single birth. Uncontradicted evidence at trial showed that two units were used because in most cases the physicians regarded a single unit as insufficient for an adult transplantation.

[*1352] PharmaStem introduced similar statements from defendant CorCell, Inc. In particular, PharmaStem pointed to a statement in CorCell's promotional literature that if cord blood could be saved, "it [**18] would be a perfect match for the donor, but could also provide life saving benefits for siblings, and other family members." Several other statements by CorCell were to the same effect--that cord blood could potentially be of benefit not only to the child but also to other members of the child's family. As in the case of CBR, however, those statements did not constitute representations that single units of CorCell's preserved cord blood would contain a sufficient number of stem cells to reconstitute an adult. PharmaStem notes in passing that one sample of CorCell's preserved cord blood was used in an adult transplantation, but the evidence at trial showed that the adult transplant did not graft and the patient died. Accordingly, that evidence provides no support at all for PharmaStem's theory of infringement.

With respect to defendant Cryo-Cell International, Inc., PharmaStem again introduced statements from the company's website that cord blood is a source of stem cells for the child or "possibly" other family members. PharmaStem's expert witness, Dr. Hendrix, interpreted that statement to refer to adult family members and to constitute a representation that each unit of cord blood [**19] preserved by Cryo-Cell contains enough stem cells to reconstitute an adult. The statements about possible use for other family members, however, do not amount to representations that any single stored unit would be sufficient by itself to reconstitute an adult, much less that all of the samples have that capacity.

Similarly, PharmaStem introduced evidence that defendant ViaCell, Inc., had advertised that cord blood could be stored "for potential use by a sibling, parent, first cousin or the newborn itself." While ViaCell's promotional materials stated that cord blood had been used in adult transplantation efforts, PharmaStem points to no representation by ViaCell that a single unit of its stored cord blood had ever been successfully used to effect hematopoietic reconstitution of an adult.

B

In addition to the evidence of the defendants' statements, PharmaStem also relied on evidence that each of the defendants tested their cord blood samples before cryopreserving them. Like the defendants' statements, however, that evidence also failed to establish that the preserved samples contained sufficient numbers of stem cells to effect hematopoietic reconstitution of an adult. The testing evidence [**20] showed that the defendants used various means to screen the cord blood samples before submitting them for cryopreservation. Those tests included determining whether the samples contained more than a minimum volume of blood, whether the samples were free of contamination, and whether they contained a minimum number of viable nucleated cells. Each of those testing measures was designed to increase the likelihood that the cord blood units contained viable stem cells and could be therapeutically useful. That evidence did not show, however, that the testing excluded all samples that lacked the capacity to reconstitute an adult, because there was no showing that the defendants chose to preserve only those samples that contained sufficient stem cells for adult reconstitution, much less that their testing procedures had that effect. Nor did PharmaStem argue that the defendants' tests could be used to show that some subset of all of the preserved samples contained enough stem cells to reconstitute an

adult. To the contrary, the evidence showed that the defendants saved cord blood samples when the defendants thought the samples [*1353] might be of some potential therapeutic use, which would include [**21] transplantation of an infant or a young child.

C

In its brief on appeal, PharmaStem refers to two pieces of scientific evidence introduced at trial that PharmaStem contends support its claim of infringement of the *'681 patent*. The first is a paper published in 2001 in the New England Journal of Medicine regarding the use of umbilical cord blood in adult transplantations. That paper was cited in promotional materials of CBR and CorCell. Although the paper showed that cord blood could have restorative effects for adults, it did not disclose whether any or all of the transplantations consisted of only a single cord blood unit. The paper therefore did nothing to prove how often a single cord blood unit from a single infant is sufficient for adult reconstitution. For that reason, the 2001 paper provided no evidentiary basis from which to infer that the particular cord blood samples preserved by any of the defendants contained a sufficient quantity of stem cells for adult reconstitution.

A second piece of scientific evidence featured by PharmaStem is a 2003 publication by the federal Food and Drug Administration reporting that an advisory committee studying cord blood transplantations had recommended [**22] that physicians be permitted to conduct adult transplantations "as long as the stem cell dose is adequate." That evidence is likewise not probative of infringement because the report makes no reference to whether a single unit of cord blood would be used in such transplantations. In fact, the transplant physician who made the presentation that led to the advisory committee's recommendation explained at trial that his recommendation against limiting transplants by age was "[b]ecause we could do cord blood transplants using two cord blood transplant [units]."

Thus, neither of the scientific exhibits cited by PharmaStem addresses whether a single cord blood unit from a single infant is sufficient to reconstitute an adult's hematopoietic system. Moreover, and significantly, neither addresses the critical question whether the particular samples preserved by the defendants contained sufficient stem cells for that purpose. Those two pieces of scientific evidence therefore do not overcome the problem with PharmaStem's evidence that the district court pointed out--that while PharmaStem may have demonstrated that the preserved cord blood units had significant therapeutic uses, and while cord [**23] blood in some amounts could be used to treat adults, the evidence was not sufficient to show that the particular cord blood units stored by the defendants contained sufficient numbers of stem cells to reconstitute the hematopoietic system of a human adult.

D

PharmaStem's failure to establish that any of the preserved cord blood samples contained sufficient stem cells to reconstitute an adult was not merely a technical flaw in its proof. The evidence at trial showed that the great majority of cord blood transplantations between the first successful transplantation in 1988 and the time of trial had been in children. Indeed, it was not until 1995 that a cord blood transplant was even attempted in an adult. The evidence also showed that more than a single unit of cord blood was used for most cord blood transplants performed on adults; the single unit collected at an individual's birth was frequently regarded as insufficient to effect hematopoietic reconstitution of an adult.

[*1354] In support of its infringement claim, PharmaStem points out that each of the defendants provided a small number of cord blood units to transplant physicians for use in transplantation procedures. The evidence shows that [**24] the four defendants had provided a total of 33 units of cord blood to transplanters by the time of trial. For the most part, however, that evidence did not distinguish between transplantations of children and transplantations of adults. To the extent that the evidence distinguished between the two, it showed that most of the supplied samples were used for transplantations of children. Moreover, with respect to the adult transplantations, PharmaStem has not pointed to any evidence that even a single transplanted cord blood unit from one of the defendants resulted in the successful reconstitution of the hematopoietic system of an adult. Thus, the evidence regarding the transplants generally, and the defendants' experience with transplants in particular, provides no basis from which to infer that some or all of the cord blood units preserved by the defendants must have contained a sufficient number of stem cells for adult reconstitution. For that reason, the district court was correct to hold that the evidence was insufficient to support the jury's verdict of infringement of the *'681 patent*.

Contrary to PharmaStem's contention, the district court's ruling did not convert a determination [**25] as to damages into a ruling on liability. Because of the manner in which PharmaStem sought to prove infringement, it committed itself to a course that had "all-or-nothing" consequences. The district court was correct to conclude that, having chosen not to try to prove that particular cord blood samples or categories of samples contained sufficient stem cells to effect hematopoietic reconstitution of an adult, PharmaStem took the risk that the court would conclude that it had failed to prove that any of the defendants' cryopreserved samples infringed. The

district court's narrow disposition of the JMOL issue simply held PharmaStem to the consequences of the strategy it adopted at trial.

E

In reaching this conclusion, we reject PharmaStem's contention that the district court abused its discretion when it determined, following the trial, that the infringement opinion of PharmaStem's expert witness Dr. Hendrix should have been struck. The district court found her testimony unhelpful to the jury, and not an appropriate subject for expert evidence, because it consisted almost entirely of her quoting from the promotional information and other materials in which the defendants described their [**26] business operations for potential customers and investors, and drawing inferences from those materials. The district court did not abuse its discretion in concluding that the jury was fully capable of understanding those materials without expert assistance and that Dr. Hendrix's testimony should have been excluded. See *General Electric Co. v. Joiner, 522 U.S. 136, 141, 118 S. Ct. 512, 139 L. Ed. 2d 508 (1997)* (abuse of discretion standard applies to district court's decision to exclude expert testimony).

Dr. Hendrix concluded from those materials that the defendants had in effect admitted that all of the cord blood samples that the defendants preserved contained a sufficient quantity of stem cells to reconstitute an adult. In particular, Dr. Hendrix interpreted the defendants' statements about their processes for preserving cord blood samples to mean that each of them tested the samples "to determine if there is a sufficient amount of cells for reconstitution for an adult. And then after that time, they cryopreserve it for storage." She admitted that she did not examine the [*1355] data obtained by the defendants from their testing of the samples; that she did not know how many, if any, successful adult transplantations had [**27] been done with cord blood samples preserved by any of the defendants; and that she did not know whether, when the defendants tested the samples, they determined whether the samples were "sufficient for an adult or sufficient for a child or sufficient for any purpose." In sum, Dr. Hendrix admitted that a particular company's decision to store a particular sample did not necessarily mean the sample was sufficient to reconstitute an adult. Nonetheless, she maintained that "[i]f the cord bloods are being stored, and the companies promise that--I mean they state in their websites that there are sufficient cells that they make available for transplantation, pediatric, sibling, older and adults, then I believe that there is the potential in all of those samples that are stored in frozen sanctuary to provide that service."

There are two problems with Dr. Hendrix's testimony, as the district court pointed out. First, because her testimony was almost entirely based on an interpretation of the defendants' marketing materials and materials directed to investors, any expertise on Dr. Hendrix's part as a cell biologist was of no apparent help to the jury. Whether or not the materials constituted [**28] admissions by the defendants that some or all of the preserved samples contained enough stem cells to reconstitute an adult was not a matter as to which Dr. Hendrix's expertise was of any apparent use. See *Daubert v. Merrell Dow Pharms., Inc., 509 U.S. 579, 592, 113 S. Ct. 2786, 125 L. Ed. 2d 469 (1993)* (admission of expert testimony "is premised on an assumption that the expert's opinion will have a reliable basis in the knowledge and experience of his discipline"). Second, not only was her expertise not necessary or useful to interpret the defendants' materials, but her interpretation was not a reasonable one. Nowhere did the defendants represent that any of the preserved cord blood samples (much less all of them) contained a sufficient number of stem cells to reconstitute an adult. The representations that the cord blood was of potential use not only for infants and children but also for adults falls significantly short of a representation that the individual cryopreserved cord blood samples each contained enough stem cells to reconstitute an adult.

To be sure, Dr. Hendrix stated in conclusory terms that she relied for her opinion not only on the defendants' materials, but also on scientific literature, testimony of [**29] experts, and the depositions of representatives of the defendants. She made clear, however, that her opinion was based principally on the assertions by the defendants that the preserved cord blood had potential uses for adults as well as for children. Moreover, Dr. Hendrix did not explain how her reliance on any of the other sources of information supported her inference about whether the defendants' preserved samples contained an infringing quantity of stem cells.

In short, we agree with the trial court that the defendants' materials did not constitute sufficient proof of infringement of the *'681 patent* and that those materials did not become proof of infringement when Dr. Hendrix read those materials back to the jury from the witness stand. There was therefore nothing in Dr. Hendrix's testimony that sufficed to remedy the insufficiency that the district court pointed out in PharmaStem's other evidence of infringement of the *'681 patent*.

IV

With respect to infringement of the *'553 patent*, the issue presented to us is again a narrow one. There is no dispute that in the 33 instances in which the defendants' cord blood samples were used in [*1356] transplant procedures, samples of cord blood containing [**30] stem cells were collected, cryopreserved, thawed, and introduced into the patient's body. In no case, however,

were all those steps performed by the same party. Instead, the defendants were typically responsible for collecting and cryopreserving the cord blood samples, while transplant physicians unrelated to the defendants thawed the cord blood and used it for transplanting.

In light of the fact that the defendants did not perform all the steps of the patented method, PharmaStem based its claim of infringement of the *'553 patent* on the theory of contributory infringement. The district court instructed the jury on contributory infringement and gave the jury special verdict questions that directed the jury's inquiry to the requirements of that theory.

The court instructed the jury that in order to prove contributory infringement, PharmaStem was required to prove, inter alia, (1) that the defendants "sold or offered to sell cryopreserved cord blood to a transplanter" and (2) that the cryopreserved cord blood that was "sold or offered for sale by the defendant was used by a single entity, or alternatively, by a group of entities that are acting in concert or working together to complete [**31] the process of infringement." The pertinent special verdict questions corresponding to those instructions required the jury to find that "the defendants and the transplant physicians are acting in concert or working together to complete the process of infringement" of the asserted claims of the *'553 patent* (special verdict question 4) and that the defendants "contributorily infringed the *'553 patent* by selling or offering to sell cryopreserved cord blood that was actually used by a third party in the direct infringement" of any of the asserted claims (special verdict question 5).

PharmaStem's theory of contributory infringement was based on the contributory infringement section of the Patent Act, [HN2] *35 U.S.C. § 271(c)*, which provides: "Whoever offers to sell or sells . . . a component of a patented machine, manufacture, combination or composition, or a material or apparatus for use in practicing a patented process, constituting a material part of the invention, knowing the same to be especially made or especially adapted for use in an infringement of such patent" shall be liable as a contributory infringer. The jury found in PharmaStem's favor on each of the special verdict questions pertaining [**32] to contributory infringement and accordingly returned a verdict of liability against all of the defendants on the *'553 patent*.

The issue on appeal is whether there was substantial evidence to support the jury's finding, in response to special verdict question 5, that each of the defendants "contributorily infringed the *'553 patent* by selling or offering to sell cryopreserved cord blood that was actually used by a third party in . . . direct infringement" of that patent. The district court ruled that the evidence was sufficient to show that the defendants sold a service to families of newborn infants (collection, processing, and cryopreservation of the newborn's umbilical cord blood), but not to show that they sold the cord blood units themselves, which belonged to the families throughout, and certainly not to show that the defendants sold the cord blood units to the transplanters.

The district court construed the contributory infringement statute to require a sale or an offer of sale of a product; the statute is not satisfied, the court ruled, by the provision of a service for compensation. Because liability under *section 271(c)* "is clearly dependent upon the accused infringer's selling [**33] or offering to sell a component of the patented process, here cord [*1357] blood units," the court held that the jury's verdict on contributory infringement could not stand, and it therefore granted the defendants' JMOL motions with respect to the *'553 patent*.

In challenging the district court's ruling, PharmaStem first argues that the jury could properly characterize as a "sale" the transaction in which the defendants obtained unprocessed umbilical cord blood, converted it into a therapeutically useful, cryopreserved cord blood product, and later provided it to transplant physicians at the behest of the client family. While cord blood is certainly a product, the transaction between the defendants and their clients is plainly not the sale of "a material or apparatus for use in practicing a patented process," as is required by *section 271(c)* with respect to method patents. The evidence at trial showed that the cord blood remained the property of the families throughout the period in which the defendants stored it. The defendants were never owners of the blood, but instead were merely bailees; they were not free to dispose of the blood as they chose, but were contractually obligated to preserve [**34] it pending the families' need for it at some point in the future. On those occasions when the cord blood was needed, the defendants provided the blood to transplanters in satisfaction of their contractual obligation to ship the families' cord blood samples to a transplanter upon direction. Neither that transaction nor any earlier transaction between the families and the defendants constituted a "sale" of the cord blood. See *Sturm v. Boker, 150 U.S. 312, 329-30, 14 S. Ct. 99, 37 L. Ed. 1093 (1893)* ([HN3] "The recognized distinction between bailment and sale is that, when the identical article is to be returned in the same or in some altered form, the contract is one of bailment, and the title to the property is not changed. On the other hand, when there is no obligation to return the specific article, and the receiver is at liberty to return another thing of value, he becomes a debtor to make the return, and the title to the property is changed."). Rather, as the trial court held, the transaction between the families and the defendants constituted the provision of a service for a fee.

In the alternative, PharmaStem argues that even if the district court was correct to characterize the defendants' activities as providing [**35] a service rather than selling a product, the court still should have upheld the jury's verdict of contributory infringement. In this regard, PharmaStem argues that *section 271(c)* is not limited to the sale of a product, but extends to the sale of a service.

PharmaStem's argument is contrary to both the language and the legislative history of *section 271(c)*. [HN4] The statute provides, in pertinent part, that a contributory infringer is one who "offers to sell or sells within the United States a patented machine, manufacture, combination or composition, or a material or apparatus for use in practicing a patented process." Although that language describes in various different ways the items that may be sold for purposes of creating liability for contributory infringement, all of the descriptions refer to the sale of a product of some sort; none of them refer to the provision of a service. Under the plain language of the statute, a person who provides a service that assists another in committing patent infringement may be subject to liability under *section 271(b)* for active inducement of infringement, but not under *section 271(c)* for contributory infringement.

The legislative background of *section 271(c)* [**36] makes clear that the district court was correct to construe that statute as confined to its plain terms. Prior to the 1952 Patent Act, no statute defined contributory infringement. Instead, as a result of court decisions, infringement was divided into two categories: "direct infringement," [*1358] which was the unauthorized making, using, or selling of the patented invention, and "contributory infringement," which was "any other activity where, although not technically making, using, or selling, the defendant displayed sufficient culpability to be held liable as an infringer." *Hewlett-Packard Co. v. Bausch & Lomb Inc., 909 F.2d 1464, 1469 (Fed. Cir. 1990)*. The 1952 Act did not make a substantive change in the law of contributory infringement, but it divided the judicially created category of contributory infringement into two statutory subsections, *section 271(b)* (inducement of infringement) and *section 271(c)* (contributory infringement). The most common type of pre-1952 contributory infringement cases were those in which "a seller would sell a component that was not covered by the claims of a patent but which had no other use except the claimed product or process." Id. That form of contributory [**37] infringement was codified in *section 271(c)*. Id.

The Senate Report on the 1952 Act confirms that *section 271(c)* was intended to deal with a particular subset of what had previously been considered contributory infringement, consisting of cases in which a party sells a particular component that is known to be intended for an infringing use and is useful only for infringement. The Senate Report states that *section 271(b)* recites "in broad terms that one who aids and abets an infringement is likewise an infringer" whereas *section 271(c)* deals specifically with the most common form of contributory infringement and "is much more restricted than many proponents of contributory infringement believe should be the case." S. Rep. No. 89-1959, at 8, 28 (1952) (characterizing *section 271(c)* as applying to "one who sells a component part of a patented invention or material or apparatus for use therein"), reprinted in 1952 U.S.C.C.A.N. 2394, 2402, 2421; see also *Jones v. Radio Corp. of Am., 131 F. Supp. 83, 83 (S.D.N.Y. 1955)* (in light of legislative history of 1952 Act, *section 271(c)* does not apply if the defendant did not sell a component of the patented combination).

In summary, the district court [**38] correctly concluded that the defendants did not sell a product and that what they provided to customers was a service for compensation. The evidence showed that the cord blood the defendants collected and preserved was never their property; instead, it remained the property of the families who engaged their services. The defendants were never the owners of the blood and thus never "sold" the blood to the families when it was needed. The district court therefore properly held that the defendants could not be found liable for contributory infringement under *section 271(c)*. [1]

1 The parties and the district court discussed the issue of joint infringement in the context of determining whether there was infringing conduct sufficient to serve as a predicate for a finding of contributory infringement. PharmaStem did not argue before the district court, and does not argue here, that liability could be premised on a theory of "joint" or "divided" infringement, even in the absence of a finding of contributory infringement under *35 U.S.C. § 271(c)*. Under that theory, two related parties are both deemed liable for direct infringement of a method patent when each performs some steps of the claimed [**39] method. The viability and scope of that theory of liability is a subject of considerable debate; it has been addressed in a number of district court cases, adverted to in a few of this court's cases, and discussed at some length by commentators. See *On Demand Mach. Corp. v. Ingram Indus., Inc., 442 F.3d 1331, 1334 (Fed. Cir. 2006)*; *Cross Med. Prods., Inc. v. Medtronic Sofamor Danek, Inc., 424 F.3d 1293, 1311 (Fed. Cir. 2005)*; Kristin E. Gerdelman, Subsequent Performance of Process Steps by Different Entities: Time to Close Another Loophole in U.S. Patent Law, *53 Emory L.J. 1987 (2004)*; Mark A. Lemley et al., Divided Infringement Claims, 33 AIPLA Q.J. 255 (2005);

Sriranga Veeraraghavan, Joint Infringement of Patent Claims: Advice for Patentees, 23 Santa Clara Computer & High Tech L.J. 211 (2006). That issue is squarely presented in a case now pending before this court, BMC Resources, Inc. v. Paymentech, L.P., No. 2006-1503. In this case, PharmaStem's theory of liability was that the defendants were liable under *section 271(c)* for contributory infringement, not under *section 271(a)* for direct infringement, and PharmaStem has continued to press that theory on appeal. We therefore are [**40] not presented with the question whether the defendants could have been held liable under *section 271(a)* under a theory of joint direct infringement through their activities in conjunction with the transplanters.

[*1359] There is another reason why the jury's verdict in this case cannot stand. The court instructed the jury, without objection from PharmaStem, that it was necessary for the sale in question to be made "to a transplanter." Yet even if a sale of a service were deemed sufficient to constitute a "sale" for purposes of *section 271(c)*, there was no evidence that any of the defendants made a sale of either products or services to the transplanters. To the contrary, the evidence showed that the service the defendants provided was a service to the donor families, for which the families paid a fee, and that there was no sale of any sort by the defendants to the transplanters or any fee paid by the transplanters to the defendants. The defendants simply transferred the cord blood units to designated transplanters upon direction from the families. Such a transaction does not constitute a "sale" to a transplanter under any definition of the term "sale." Accordingly, the district court properly [**41] concluded that the jury's verdict was legally insufficient to establish infringement under the law of the case as given by the court to the jury and accepted by the parties. We therefore uphold the portion of the court's judgment granting the defendants' JMOL motions with respect to the *'553 patent*.

V

The jury returned verdicts in favor of PharmaStem on the defendants' counterclaims challenging the validity of the two patents in suit. In its opinion on the defendants' JMOL motions, the district court held that the jury's verdicts on the validity issues were supported by substantial evidence. In their cross-appeal, the defendants contest the portions of the trial court's judgment rejecting their challenges to the patents on grounds of anticipation, obviousness, and (in the case of the *'681 patent*) indefiniteness. Each of those issues presents a close question. Because we hold that the district court should have granted the defendants' motion for JMOL on the issue of obviousness, it is not necessary for us to address the defendants' arguments with respect to the issues of indefiniteness and anticipation.

A

[HN5] Obviousness is a legal conclusion that we review de novo. The statutory standard requires [**42] us to decide whether the subject matter of the claimed invention "would have been obvious at the time the invention was made to a person of ordinary skill in the art to which [the subject matter of the invention] pertains." *35 U.S.C. § 103(a)*; *Eli Lilly & Co. v. Zenith Goldline Pharms., Inc., 471 F.3d 1369, 1377 (Fed. Cir. 2006)*; *DyStar Textilfarben GmbH & Co. Deutschland KG v. C.H. Patrick Co., 464 F.3d 1356, 1360 (Fed. Cir. 2006)*. Underpinning that legal issue are factual questions relating to the scope and content of the prior art, the differences between the prior art and the claimed invention, the level of ordinary skill in the art, and any relevant secondary considerations, such as commercial success, long-felt need, and the failure of others. See *Eli Lilly, 471 F.3d at 1377*; *DyStar, 464 F.3d at 1360*; *Medichem, S.A. v. Rolabo,* [*1360] *S.L., 437 F.3d 1157, 1164 (Fed. Cir. 2006)*. [HN6] Under Third Circuit law, which in this case dictates the standard for reviewing the denial of the motion for JMOL, we review the district court's action "de novo by reapplying the JMOL standard" applied by the district court. *Seachange Int'l, Inc. v. C-COR Inc., 413 F.3d 1361, 1368 (Fed. Cir. 2005)*. Thus, [HN7] in reviewing [**43] the denial of the JMOL motion on the issue of obviousness, we examine the evidence in the light most favorable to the verdict and determine whether a reasonable jury could have found all the facts necessary to support the verdict of nonobviousness, i.e., whether substantial evidence supports the verdict. See *Caver v. City of Trenton, 420 F.3d 243, 262 (3d Cir. 2005)*; *Connell v. Sears, Roebuck & Co., 722 F.2d 1542, 1546 (Fed. Cir. 1983)*.

B

The defendants contend that the two patents in suit are invalid for obviousness based on a combination of several prior art references. [HN8] In such a case, the burden falls on the patent challenger to show by clear and convincing evidence that a person of ordinary skill in the art would have had reason to attempt to make the composition or device, or carry out the claimed process, and would have had a reasonable expectation of success in doing so. See *Medichem, 437 F.3d at 1164*; *Noelle v. Lederman, 355 F.3d 1343, 1351-52 (Fed. Cir. 2004)*; *Brown & Williamson Tobacco Co. v. Philip Morris, Inc., 229 F.3d 1120, 1121 (Fed. Cir. 2000)*; see also *KSR Int'l Co. v. Teleflex Inc., 127 S. Ct. 1727, 1740, 167 L. Ed. 2d 705 (2007)* (a combination of elements "must do more than yield a predictable [**44] result"; combining ele-

ments that work together "in an unexpected and fruitful manner" would not have been obvious).

In view of the prior art references, the first part of that test is plainly satisfied here. The idea of using cryopreserved cord blood to effect hematopoietic reconstitution was not new at the time the inventors filed the applications that matured into the *'681* and *'553 patents*. Two of the prior art references--articles by Ende and Knudtzon--suggest using cord blood for that purpose. Two others--an article by Koike and a doctoral dissertation by Vidal--suggest cryopreservation and storage of the cord blood until needed. Accordingly, this is not a case in which there is any serious question whether there was a suggestion or motivation to devise the patented composition or process.

The more difficult question is whether the prior art would have given rise to a reasonable expectation of success in creating the process claimed in the *'553 patent* and the composition claimed in the *'681 patent*. In [**45] addressing that question, the parties focus on whether the inventors had a reasonable expectation that cord blood could be successfully used in transplants for hematopoietic reconstitution.

On the question whether the inventors had a reasonable expectation of success, the district court relied principally on testimony by PharmaStem's expert, Dr. Irwin Bernstein. In testimony cited by the court, Dr. Bernstein explained that there were problems with transplant tissues that had been used previously, including bone marrow and adult blood; that those working in the transplant field did not believe blood would be suitable as a transplant tissue; and that researchers in his group were surprised at the successful result of the first transplant of cord blood into a human. That evidence, according to the court, justified the jury in finding that persons of skill in the field of hematopoietic reconstitution "would not have expected cord blood to be a successful transplant tissue." In light of that evidence and the evidence of secondary considerations such as long-felt need and commercial success, [*1361] and in light of the PTO's issuance of the patents over several of the prior art references that were [**46] in issue at trial, the court concluded that "there is no basis to overturn the jury's verdict that the Patents-In-Suit are not obvious."

The defendants argue that the prior art suggested using cryopreserved cord blood for hematopoietic reconstitution and showed that persons of skill in the field would have had a reasonable expectation that the use of cord blood in transplants would be successful. For that reason, according to the defendants, the asserted claims were obvious as a matter of law.

Like the district court, PharmaStem relies principally on Dr. Bernstein's testimony to support its argument that the asserted claims of the *'681* and *'553 patents* were not invalid for obviousness. Citing his testimony, PharmaStem argues that those skilled in the art at the time of the inventions "did not even yet know of the presence of stem cells in cord blood." PharmaStem argues that Dr. Bernstein's assertion that it was not known that cord blood contained stem cells, combined with his testimony regarding problems with transplant tissues used prior to the *'681* and *'553 patents*, shows that those in the field of hematopoietic reconstitution "would not have expected cord blood to be a successful [**47] transplant tissue."

The cornerstone of Dr. Bernstein's testimony at trial was that none of the prior art showed that cord blood contains stem cells. According to Dr. Bernstein, the presence of stem cells in cord blood was not conclusively established before the mouse studies described in the joint specification and the 1988 human cord blood transplant referred to in the specification of the *'553 patent*.

The problem with Dr. Bernstein's testimony about the prior art references is that it cannot be reconciled with statements made by the inventors in the joint specification and with the prior art references themselves. Dr. Bernstein distinguished each of the prior art references on the ground that none of them disclosed the presence of stem cells in cord blood. Even though some of the references referred to stem cells as being present in cord blood, Dr. Bernstein took the position that those statements in the prior art references reflected flawed nomenclature and that the most the data underlying the prior art references showed was that cord blood contained progenitor cells. Progenitor cells are the cells that generate several different types of cells that make up the blood and immune system [**48] but are less primitive than hematopoietic stem cells. According to Dr. Bernstein, it was not proved that stem cells, as opposed to the less primitive progenitor cells, are present in cord blood until the patentees performed the mouse experiments reported in the joint specification. Those experiments showed that relatively small amounts of fetal blood were sufficient to effect hematopoietic reconstitution in lethally irradiated mice. Dr. Bernstein added that in light of the poor results obtained with transplantations of adult blood "it had to take a leap of thinking that cord blood was different."

The joint specification, however, tells a different story. There, the inventors acknowledged that it was previously known that the properties of cord blood are quite different from those of adult blood and that hematopoietic stem cells had been found in cord blood in much greater concentrations than in adult blood. Citing a number of references, the inventors stated the following:

> A human hematopoietic colony-forming cell with the ability to generate progeni-

> tors for secondary colonies has been identified in human umbilical cord blood. In addition, hematopoietic stem cells have been demonstrated [**49] in human umbilical cord blood, by colony formation, [*1362] to occur at a much higher level than that found in the adult. The presence of circulating hematopoietic progenitor cells in human fetal blood has also been shown. Human fetal and neonatal blood has been reported to contain megakaryocyte and burst erythroblast progenitors with increased numbers of erythroid progenitors in human cord blood or fetal liver relative to adult blood.

'681 patent, col. 4, ll. 15--34 (citations omitted); *'553 patent*, col. 4, ll. 21--42 (citations omitted).

That excerpt from the specification cannot be squared with Dr. Bernstein's characterization of the prior art. Contrary to Dr. Bernstein's contention that the prior art did not disclose the presence of stem cells in cord blood, the inventors cited several prior art references and stated flatly that "hematopoietic stem cells have been demonstrated in human umbilical cord blood." Moreover, the inventors noted that the prior art references showed that the concentration of stem cells in cord blood was "at a much higher level than in the adult." Nor can those statements in the specification be dismissed as reflecting a careless use of the term "hematopoietic stem [**50] cell," i.e., the use of that term when the inventors meant to refer to progenitor cells. That is made clear by context, as the sentence that immediately follows the reference to "hematopoietic stem cells" states that "the presence of hematopoietic progenitor cells in human fetal blood has also been shown."

Accordingly, PharmaStem's argument that stem cells had not been proved to exist in cord blood prior to the experiments described in the patents is contrary to the representation in the specification that the prior art disclosed stem cells in cord blood. [HN9] Admissions in the specification regarding the prior art are binding on the patentee for purposes of a later inquiry into obviousness. See *Constant v. Advanced Micro Devices, Inc., 848 F.2d 1560, 1570 (Fed. Cir. 1988)* ("A statement in the patent that something is in the prior art is binding on the applicant and patentee for determinations of anticipation and obviousness."); *Sjolund v. Musland, 847 F.2d 1573, 1577-79 (Fed. Cir. 1988)* (patent specification admitted that certain matter was prior art, and thus "the jury was not free to disregard [that matter]" and "must have accepted [it] as prior art, as a matter of law"); *In re Fout, 675 F.2d 297, 300 (CCPA 1982)*; [**51] *In re Nomiya, 509 F.2d 566, 571 (CCPA 1975)*.

Nor is there any unfairness in holding the inventors to the consequences of their admissions, as their characterization of the prior art as showing the presence of stem cells in cord blood is hardly unreasonable. At trial, the defendants' expert acknowledged that, prior to the time of the first successful cord blood transplant, stem cells could not be conclusively proved to be present in cord blood. He explained, however, that in light of the discovery of substantial numbers of progenitor cells in cord blood--roughly equivalent to the number of such cells in bone marrow--it was appropriate for the authors of the prior art references to infer the presence of stem cells in cord blood, even though positive proof of their presence was not available.

The prior art references provide strong support for that interpretation. Mouse studies reported by Barnes in a 1964 article showed that the blood of fetal and neonatal mice contained a much greater concentration of colony-forming units (i.e., progenitor cells) than adult blood. Barnes identified the colonies in question as containing stem cells. A 1974 article by Knudtzon similarly noted that an "increased [**52] concentration of hemopoietic stem cells has been found in the blood of mouse embryos when compared to the concentration after birth." Knudtzon also conducted [*1363] tests on human umbilical cord blood, determining that the concentration of in vitro colony-forming cells in cord blood is likewise much greater than in human adult blood and that the concentration is comparable to the concentration in bone marrow tissue. Knudtzon concluded that "the finding of an increased concentration of colony-forming cells in human cord blood comparable in number with human bone marrow cultures indicates that cord blood might be used as a source of hemopoietic stem cells for the restoration of bone marrow function in humans." Two years later, a case study by Ende reported a transfusion of 45 milliliters of human cord blood into a human patient, which resulted in a temporary hematopoietic graft that lasted for five weeks. Ende cited other research indicating that a similar or even larger amount of bone marrow would be needed to achieve a successful permanent graft.

A 1978 article by Prindull noted that animal experiments showed that fetal blood contains more than 100 times as many stem cells as are present in [**53] adult blood and suggested that because the fetal hematopoietic system is in a state of physiologic proliferation, human cord blood could constitute a source of hematopoietic stem cells. An article by Koike, in 1982, described the results of freezing and thawing cells derived from bone marrow and cord blood. It showed that even immature progenitor cells can survive cryopreservation and concluded that because cord blood contains "many pluripo-

tent and nearby progenitor cells comparable to marrow cells," cord blood or other fetal tissue could be a useful source of hematopoietic progenitor cells for transplantation. In 1985, a doctoral dissertation by Vidal concluded, based on various studies, that "cord blood contains sufficient hematopoietic stem cells to effect a transplant," that "cord blood can be used for this purpose," and that "cryopreserved cord blood banks might exist."

That collection of prior art shows (1) that bone marrow transplants can result in hematopoietic reconstitution; (2) that cord blood, like bone marrow but unlike adult blood, contains large numbers of progenitor cells; and (3) that the high concentration of primitive progenitor cells in cord blood suggests that in [**54] humans, as in mice, the cells responsible for hematopoiesis migrate at about the time of birth from fetal organs to the bone marrow. Under those circumstances, it was reasonable for the inventors of the patent, like the authors of the prior art references, to infer the presence of high concentrations of stem cells in cord blood, even though the prior art studies did not offer conclusive proof of their presence.

C

Given that the jury was legally required to find that that those of skill in the art would believe that cord blood contained hematopoietic stem cells, the question before us is whether a reasonable jury could nonetheless have found the invention nonobvious. We conclude a reasonable jury could not have done so. While the inventors may have proved conclusively what was strongly suspected before--that umbilical cord blood is capable of hematopoietic reconstitution--and while their work may have significantly advanced the state of the science of hematopoietic transplantations by eliminating any doubt as to the presence of stem cells in cord blood, the mouse experiments and the conclusions drawn from them were not inventive in nature. Instead, the inventors merely used routine research [**55] methods to prove what was already believed to be the case. [HN10] Scientific confirmation of what was already believed to be true may be a valuable contribution, but it does not give rise to a patentable [*1364] invention. See *KSR, 127 S. Ct. at 1732* ("Granting patent protection to advances that would occur in the ordinary course without real innovation retards progress . . . ."); *Pfizer, Inc. v. Apotex, Inc., 480 F.3d 1348, 1367-69 (Fed. Cir. 2007)* (simply because the formation and properties of a new compound must be verified through testing does not mean that the compound satisfies the test for patentability "since the expectation of success need only be reasonable, not absolute"); *In re Merck & Co., 800 F.2d 1091, 1097 (Fed. Cir. 1986)* ("Obviousness does not require absolute predictability."). Good science and useful contributions do not necessarily result in patentability.

This court's decision in *In re O'Farrell, 853 F.2d 894, 903 (Fed. Cir. 1988)*, provides useful guidance for determining whether the expectation of success from a particular line of inquiry is great enough to render a resulting invention obvious. The court noted that [HN11] obviousness "does not require absolute predictability of success. [**56] Indeed, for many inventions that seem quite obvious, there is no absolute predictability of success until the invention is reduced to practice." *853 F.2d at 903*. On the other hand, the court explained, an invention would not be invalid for obviousness if the inventor would have been motivated "to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful." Id. Likewise, an invention would not be deemed obvious if all that was suggested "was to explore a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it." Id.; see also *Medichem, S.A. v. Rolabo, S.L., 437 F.3d 1157, 1166-67 (Fed. Cir. 2006)*.

This case is not one in which "the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful," nor is it one in which the prior art "gave only [**57] general guidance as to the particular form of the invention or how to achieve it." *O'Farrell, 853 F.2d at 903*. The prior art suggested cryopreserving cord blood from a single infant and transplanting that blood into a patient to achieve hematopoietic reconstitution. PharmaStem does not suggest, and Dr. Bernstein's testimony did not reveal, that there was an array of possible choices as to how to achieve that objective or that there were problems to be solved in implementing the prior art suggestion that were not adumbrated in the prior art. To the contrary, the joint specification indicates that each step of the cryopreservation and transplantation procedure had been spelled out in the prior art. PharmaStem does not claim that there was anything novel about the method by which it proposed to collect, cryopreserve, and transplant the cord blood. Instead, in responding to the defendants' obviousness challenge, PharmaStem focuses entirely on the purported novelty of its proof that stem cells are present in fetal blood, a demonstration that Dr. Bernstein testified was necessary to give transplant physicians sufficient confidence in the use of cord blood for hematopoietic reconstitution [**58] to try the procedure on humans. As we have explained, however, providing proof sufficient to justify conducting in vivo procedures on humans, while useful, is not a test of patentability. The evidence at trial demonstrated that the patentees did not invent a new procedure

or a new composition; instead, they simply provided experimental proof that the cord blood could be used to effect hematopoietic reconstitution [*1365] of mice and, by extrapolation, could be expected to work in humans as well.

D

In addition to its reliance on Dr. Bernstein's testimony about the prior art references, PharmaStem invokes various secondary considerations that it contends support the jury's verdict on obviousness. In particular, PharmaStem points to evidence that the inventors were widely recognized as pioneers in the use of cord blood for hematopoietic reconstitution, including statements by the defendants and their representatives. Defendant ViaCord's business plan praised the inventors as "trailblazers," and a founder of defendant Cryo-Cell wrote to the inventors' company and stated: "[N]o one will ever dispute that you, as the pioneers in the medical technology . . . will be the frontrunners in the field of [**59] utilizing the blood from the umbilical cord for restoring hematopoietic [sic] through marrow transplants." Even the defendants' expert had previously referred to the inventors as the first to suggest the use of human umbilical cord blood as a source of transplantable hematopoietic stem cells, although he disclaimed those statements at trial on the ground that he had subsequently determined that it was incorrect to give the inventors credit for conceiving the invention. The problem with that evidence is that there was no indication that the praise for the inventors' work was based on any inventive contribution they made, as opposed to their proof, through laboratory work, that fetal blood contains large numbers of stem cells. As noted, the former is a basis for patentability; the latter is not.

PharmaStem also points to Dr. Bernstein's testimony that researchers in his group in Seattle were "surprised" at the successful human cord blood transplantation in 1988. There are two problems with that evidence. First, there was no indication that either Dr. Bernstein or members of his research group were previously aware of the prior art references that laid the groundwork for the inventors' [**60] experiments. Dr. Bernstein stated that his surprise at the successful use of cord blood was based on the poor results obtained with transplants of adult blood; he did not state that the success of the human transplant would have been surprising to one familiar with the prior art references introduced at trial, including those references that featured the important differences between adult blood and cord blood as potential transplant tissues.

Second, Dr. Bernstein tied the "surprise" of his research group to the success of the 1988 human cord blood transplant, not to the results reported in the patents. Although the transplant was based on work done by the inventors, it took place long after the filing of the application for the *'681 patent* and shortly before the filing of the application for the *'553 patent*. As a result, the specification of the *'681 patent* does not refer to the 1988 transplant at all, and the specification of the *'553 patent* does not contain any account of the results of that transplant. At the time of the application for the *'553 patent*, all that was known and disclosed about the 1988 transplant was that it had been attempted.

Moreover, although it is true, as PharmaStem [**61] argued, that physicians began performing human transplants only after the inventors conducted their mouse experiments, the evidence at trial showed that physicians were reluctant to try a new procedure such as a cord blood transplant on humans without a very strong scientific basis for concluding that it was likely to work. The prior art already indicated that cord blood was likely to be a valuable source of hematopoietic stem cells; the mouse studies merely provided supporting [*1366] evidence for that conclusion, evidence that the transplant physicians regarded as sufficient to justify trying the procedure on a human child.

E

Finally, PharmaStem argues that the jury's verdict is supported by the decision of the Patent and Trademark Office ("PTO") to issue the *'681* and *'553 patents*, and to confirm the *'681 patent* following reexamination, over some of the same references that the defendants cited at trial. [HN12] When the party asserting invalidity relies on references that were considered during examination or reexamination, that party "bears the added burden of overcoming the deference that is due to a qualified government agency presumed to have done its job." *Polaroid Corp. v. Eastman Kodak Co., 789 F.2d 1556, 1560 (Fed. Cir. 1986)*; [**62] see also *Al-Site Corp. v. VSI Int'l, Inc., 174 F.3d 1308, 1323 (Fed. Cir. 1999)*.

The examiner who issued the reexamination certificate for the *'681 patent* summarized her analysis of the prior art by stating that none of the cited references "addresses the presence of hematopoietic stem cells in umbilical cord or placental blood, that these cells may successfully be cryopreserved, or that, as a collection from a single human at birth, these cells may comprise an amount that is sufficient to effect hematopoietic reconstitution of a human adult." That explanation is flawed for three reasons. First, as we have explained, the prior art references and the admissions in the specification address the presence of hematopoietic stem cells in cord blood, even though the references may not conclusively prove their presence. Second, Koike established that cord blood could be cryopreserved without substantial losses in the population of progenitor cells; the inventors con-

tributed nothing more with respect to cryopreservation, as their mouse experiments were not performed with cryopreserved blood. Third, while the joint specification states that the amount of cord blood obtained at the time of birth [**63] would often be sufficient to transplant an adult, the inventors reached that conclusion simply by comparing the known properties of bone marrow against the results of routine testing of their own cord blood samples.

The specification explains that, because of the inability to determine the number of stem cells present in a particular composition, researchers and transplanters use surrogate assays from which they can infer that stem cells are present and in roughly what numbers. One of the surrogate assays that the joint specification describes in detail and that was the subject of testimony at trial is the assay for CFU-GM (colony-forming units for granulyte and macrophage cells), i.e., progenitor cells that produce the more specialized granulyte and macrophage cells. The inventors compared the results of conventional CFU-GM assays of cord blood samples with published reports of the number of CFU-GM in bone marrow samples sufficient for successful hematopoietic reconstitution. *'681 patent*, col. 50, line 64, to col. 51, line 15; *'553 patent*, col. 51, ll. 44-68. Thus, the inventors reported that prior art studies showed that in cases involving autologous bone marrow transplants, "rapid [**64] repopulation of hematopoiesis in patients with acute leukemia was associated with as few as 0.25 million progenitor cells

> [CFU-GM]." *'681 patent*, col. 13, ll. 49--54. The inventors' assays of cord blood samples, confirmed by prior art studies, showed that 50 milliliters of cord blood would contain up to more than 0.5 million CFU-GM. Id., col. 13, ll. 55--63. Thus, the inventors' conclusion that a single unit of cord blood can result in hematopoietic reconstitution of an adult was simply the result of a comparison between the well-known properties of bone marrow and their own conventional [*1367] assays of a number of samples of cord blood.

In sum, while the issue of obviousness in this case presents us with a difficult question in light of the standards of proof and review that are applied to an appellate challenge to a jury verdict of nonobviousness, we are persuaded that there was clear and convincing evidence that the asserted claims of the *'681* and *'553 patents* would have been obvious and that it was unreasonable for the jury to reach the opposite conclusion. We therefore reverse the denial of JMOL on that issue and remand to the district court for entry of judgment in the defendants' [**65] favor.

VI

This was a closely contested case both at trial and on appeal, and the JMOL motions presented the district court with an unusually difficult set of challenges. We are satisfied that the district court correctly resolved each of the issues that the parties have raised and we have addressed on appeal, with the sole exception of the cross-appeal on the issue of obviousness. We therefore affirm the judgment of the district court with respect to the appeal but reverse the judgment on the cross-appeal with respect to the issue of obviousness. As to that issue, we reverse and remand to the district court for entry of judgment in the defendants' favor.

Each party shall bear its own costs for this appeal and cross-appeal.

AFFIRMED IN PART, REVERSED IN PART, and REMANDED.

DISSENT BY: NEWMAN

DISSENT

NEWMAN, Circuit Judge, dissenting.

I respectfully dissent. After a three week trial the jury sustained the validity of these patents, the district court in a thorough opinion upheld the verdicts of validity, and validity was confirmed in three reexaminations by the Patent and Trademark Office. Today my colleagues on this panel hold that the inventions in the *'681 patent* and its continuation-in-part the *'553 patent* [**66] are obvious to them, and not infringed.

The undisputed evidence at trial was that these long-sought life-saving inventions were achieved amid general scientific skepticism, despite the extensive research that was being conducted by many scientists in this field, as set forth in the patents in suit. The discoveries of these inventors were met with universal acclaim and widespread utilization, including the founding of many commercial enterprises, all of which are reported to have licensed the patents except for these defendants. Unimpressed by these considerations, my colleagues on this panel now reconstruct these inventions by selection and inference, with perfect hindsight of the discoveries. The evidence at trial was that this achievement eluded persons working in the field, despite speculation concerning its potential and recognition of its value if it could actually be achieved; despite the powerful interest in such a life-saving advance. Instead, my colleagues simply reweigh selectively extracted evidence, ignore the actual peer response and acclaim at the time these inventions

were made, and decide that this long-sought advance would have been obvious to this court.

Inventors [**67] Edward A. Boyse, Hal E. Broxmeyer, and Gordon W. Douglas made possible a new industry with PharmaStem's predecessor Biocyte, Inc., founded by the inventors. The record contains many publications reporting the work of these inventors, and the evidence was undisputed that they were the first to achieve the transplantation of umbilical cord stem cells for reconstitution of the human hematopoietic system. Although my colleagues manage to reconstruct this extensive scientific effort as simple routine that is obvious to judges, the processes of discovery in [*1368] complex science make it particularly necessary to view the achievement in the context of the knowledge at the time the invention was made, and to judge it as it was judged by scientific peers at that time, with the assistance of the hard fact of commercial success in a field in which the need was great and success had long been eluded. See *Graham v. John Deere Co., 383 U.S. 1, 17-18, 86 S. Ct. 684, 15 L. Ed. 2d 545 (1966)* ("Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented.") (quoted in *KSR International Co. v. Teleflex Inc., 127 S. Ct. 1727, 1734, 167 L. Ed. 2d 705 (2007)).*

The [**68] panel majority scours the prior art for clues that could fit the eventual achievement, and then rules that the achievement was obvious, no matter that it eluded the others whose work is now compiled by this court so as to invalidate these patents. The "prior art" selected by my colleagues spans many years of scientific interest and effort, yet the ultimate discovery of the presence of stem cells along with or instead of progenitor cells, the successful preservation of these cells, the extensive experimentation with transplantation into animal models and ultimately into humans, and the successful hematopoietic reconstitution of blood that has been destroyed by disease or radiation, was not achieved in the prior art. The judicial determination of "obviousness" should be made in the context of the state of knowledge at the time these inventions were made. Nor should the courts lose sight of the powerful policy that underlies the patent law, whereby recognition and protection of technological and scientific advance is legally established in order to serve the public interest in having the benefit of such advance through economic enterprise.

My colleagues ignore not only the scientific experts [**69] who testified at the trial, but also the PTO examiners who conducted the three reexaminations. In *Dickinson v. Zurko, 527 U.S. 150, 119 S. Ct. 1816, 144 L. Ed. 2d 143 (1999)* this court was reminded of its obligation to give appropriate deference to agency expertise, including that of the PTO. The references that are analyzed by the panel majority, in its sua sponte finding of obviousness, were before the PTO for examination and multiple reexaminations. My colleagues do not explain where the PTO went wrong; instead, they rearrange the past, criticize the acclaim heaped on these inventors, and propose that if the people in this field knew what this court knows, they would not have been so impressed.

To the contrary: the acclaim sounded by even these defendants is a powerful testament to how this invention was viewed. From my colleagues' invalidation of these patents on the ground of obviousness, reversing the jury verdict, I respectfully dissent. I must also dissent from the rejection of the jury verdict of infringement, for the district court applied a new and incorrect evidentiary standard that does not warrant ratification.

THE VALIDITY ISSUES

The jury's special verdicts upholding patent validity were sustained by the [**70] district court on post-trial motions. The defendants raised the ever-present multiple grounds of attack that appear in patent cases, and cross-appeal the jury verdicts on the issues of anticipation, indefiniteness, and obviousness, but do not appeal the verdicts for the plaintiff on the issues of inventorship, inequitable conduct, and antitrust violation. My colleagues reverse the jury verdict of unobviousness, and decline to reach the verdicts upholding validity on the issues of anticipation and indefiniteness. [*1369] The district court sustained each of these verdicts. These issues were also raised for multiple reexaminations, and the PTO upheld patent validity on these grounds.

The teaching of *Cardinal Chemical Co. v. Morton International, Inc., 508 U.S. 83, 97, 113 S. Ct. 1967, 124 L. Ed. 2d 1 (1993)* ("[T]he Federal Circuit is not a court of last resort. If that court had jurisdiction while the case was pending before it, the case remains alive (barring other changes) when it comes to us. The Federal Circuit's determination that the patents were not infringed is subject to review in this Court, and if we reverse that determination, we are not prevented from considering the question of validity merely because a lower court [**71] thought it superfluous."), strongly encourages our appellate review of the major issues that were decided and appealed, if such issues would be relevant to patent validity upon further proceedings in the Court. Review of the issues of validity that were litigated sheds further light on the nature of the invention; leaving these issues in silent limbo, despite the elaborate trial and appellate briefing and argument of these issues, distorts the context of the jury verdicts as well as the reexaminations. In this context I discuss the several issues of validity that are appealed, and explain why their judgment also warrants affirmance.

Anticipation

The jury found that the patents had not been proven invalid on the ground of anticipation. "Anticipation" means lack of novelty; that is, that the invention was already known. It is a factual question whose finding, when tried to a jury, is reviewed for support by substantial evidence on the record as a whole. *Acromed Corp. v. Sofamor Danek Group, Inc., 253 F.3d 1371, 1378-79 (Fed. Cir. 2001)*; *Advanced Display Sys. v. Kent State Univ., 212 F.3d 1272, 1281 (Fed. Cir. 2000)*.

A patent claim is deemed anticipated when every element and limitation [**72] of the claim is found in a single prior art reference, either explicitly or inherently. *Dayco Products, Inc. v. Total Containment, Inc., 329 F.3d 1358, 1368 (Fed. Cir. 2003)*. In order to anticipate, the reference must place a person who has ordinary skill in the field of the invention, in possession of the invention. See *Akzo N.V. v. United States Int'l Trade Comm'n, 808 F.2d 1471 (Fed. Cir. 1986)* ("anticipation requires that each and every element of the claimed invention be disclosed in a prior art reference. In addition, the prior art reference must be enabling, thus placing the allegedly disclosed matter in the possession of the public.")

The reference on which the defendants rely for anticipation is an article by Kenichi Koike entitled "Cryopreservation of Pluripotent and Committed Hemopoietic Progenitor Cells from Human Bone Marrow and Cord Blood," 25 Acta Paediatrica Japonica 275 (1983). Koike describes the preservation, by freezing in liquid nitrogen, of pluripotent and progenitor cells of bone marrow and umbilical cord blood, and shows that these cells retain much of their progenitor activity upon thawing. Koike does not mention stem cells, and states that "hematopoietic progenitor [**73] cells, especially pluripotent progenitor cells are the most important to repopulate the bone marrow." Id. at 276. Koike concludes with the suggestion that fetal cells or organs may be a source of progenitor cells for marrow transplantation, in the following statement:

> [T]he results that cord blood cells contain many pluripotent and nearby progenitor cells comparable to marrow cells, indicate that fetal hematopoietic cells or organs may be useful as one of [*1370] the sources of hematopoietic progenitor cells for marrow transplantation.

Koike at 281.

The defendants argued at trial, and repeat on this appeal, that even if stem cells were not known or shown by Koike to be present in umbilical cord blood, the claims are "inherently" anticipated by Koike because stem cells were present even if unknown. PharmaStem responded that inherent anticipation is avoided by lack of recognition, by lack of enablement, and by the limitations in the claims, including for the *'681* claims the limitations to therapeutic compositions and the requirements that the cryopreserved cord blood units contain sufficient stem cells to reconstitute an adult. These aspects were extensively probed at the trial, and witnesses explained [**74] the various claim limitations and the prior art.

The district court, on post-trial motions, held that the jury verdict that the claims are not anticipated was supported by substantial evidence. The court referred to testimony of the expert witnesses for both sides, who agreed that Koike did not show hematopoietic reconstitution using cord blood, and that Koike did not enable transplantation. The defendant's expert witness testified (on cross-examination) that Koike's small samples could not contain a therapeutic amount of stem cells, and that the Koike article does not reflect knowledge of stem cells or indicate their presence to persons of skill in the field or show how to achieve transplantation of cord blood cells. As explained in *In re Donohue, 766 F.2d 531, 533 (Fed. Cir. 1985)*, possession of the invention adequate to show anticipation requires that a person of ordinary skill in the field of the invention would discern every element of the invention in the allegedly anticipating reference, and know how to carry it out based on the state of knowledge at the time of the reference. See, e.g., *Elan Pharms., Inc. v. Mayo Found., 346 F.3d 1051, 1054 (Fed. Cir. 2003)* (a claim "cannot [**75] be anticipated by a prior art reference if the allegedly anticipatory disclosures cited as prior art are not enabled"). There was substantial evidence that Koike did not establish that there were stem cells in umbilical cord blood nor teach a therapeutic composition for use in hematopoietic reconstitution of a human adult.

The *'681 patent* describes the prior art in detail, including the following with respect to stem cells in human umbilical cord blood:

> A human hematopoietic colony-forming cell with the ability to generate progenitors for secondary colonies has been identified in human umbilical cord blood (Nakahata, T. and Ogawa, M., 1982, J. Clin. Invest. 70:1324-1328). In addition, hematopoietic stem cells have been demonstrated in human umbilical cord blood, by colony formation, to occur at a much higher level than that found in the adult (Prindull, G., et al., 1978, Acta Paediatr. Scand. 67:413-416; Knudtzon, S., 1974, Blood 43(3):357-361).

'681 patent, col. 4, lines 15-24. The *'681 patent* explains that the differences between stem and progenitor cells are operational and depend on functional rather than on morphological criteria. Col. 3, lines 4-39. In functional assays, stem cells [**76] can be identified by spleen colony forming units (CFU-S), whereas multipotent progenitor cells can be identified through colony-forming unit-granulocyte, erythrocyte, monocyte/macrophage, megakaryocyte (CFU-GEMM) relatively differentiated progenitor cells through colony-forming unit-granulocyte, macrophage (CFU-GM) and burst-forming unit-erythroid (BFU-E). Id. at col. 26, lines 1-16. Koike, in determining the viability of the cryopreserved fetal bone marrow and cord blood, [*1371] employed CFU-GM and BFU-E assays to measure progenitor cells, not stem cells.

The patent examiner concluded, and witnesses at trial testified, that the Koike reference is directed to progenitor cells, not stem cells. The reexamination record was in evidence, wherein the examiner stated:

> The remaining references that recited umbilical cord blood, specifically the Koike and Vidal references, recited the cryopreservation of a Ficoll-Hypaque fraction of umbilical cord blood and did not provide any evidence that viable human neonatal or fetal hematopoietic stem cells were present in the thawed samples.

Notice of Intent to Issue Reexamination Certificate at 4 (Jan. 11, 2000). The examiner observed that Koike did not mention [**77] stem cells and did not show or enable transplantation to an adult, and that although Koike postulated that cord blood may be a source of hematopoietic progenitor cells, Koike did not show how or if such use could be achieved. The examiner's reasons for allowance included the following:

> . . . Since hematopoietic stem cells engage in both replication and differentiation, the presence of progenitors (differentiated stem cells) is not predictive of the presence of stem cells. All of the prior art references which taught the cryopreservation of a Ficoli-Hypaque fraction of umbilical cord blood assayed for the presence of progenitor cells and merely theorized on the presence of stem cells. None of the prior art references demonstrated the presence of stem cells in the umbilical cord blood.

Id. When the reference relied on at trial was before the patent examiner, a reasonable jury may give weight to the examiner's view of the reference when deciding whether invalidity has been proved by clear and convincing evidence. See *Hewlett-Packard Co. v. Bausch & Lomb Inc., 909 F.2d 1464, 1467 (Fed. Cir. 1990)* (referring to the particularly heavy burden in establishing invalidity on the same prior art [**78] that was examined in the PTO).

The defendants argue that it is irrelevant whether Koike described or recognized the presence of stem cells in cord blood, because they were inherently there. However, as discussed in *Turbo Care Div. Of Demag Delaval Turbomachinery Corp. v. General Electric Co., 264 F.3d 1111, 1119 (Fed. Cir. 2001)*, "[i]n order for a disclosure to be inherent, 'the missing descriptive matter must necessarily be present in the [original] applicant's specification such that one skilled in the art could recognize such a disclosure," (quoting *Tronzo v. Biomet, Inc., 156 F.3d 1154, 1159 (Fed. Cir. 1998))*. As the district court pointed out and as the expert witnesses testified, Koike does not show the claim limitations to therapeutic compositions or that the cryopreserved blood units must be from a single human or that stem cells must be present in an amount sufficient for hematopoietic reconstitution of a human adult, or suggest how to conduct a successful transplantation. Witnesses testified that persons in this field of science did not have the knowledge to routinely fill these omissions, and reinforce the examiner's statement that "the presence of progenitors (differentiated [**79] stem cells) is not predictive of the presence of stem cells." See Reexamination Notice of Intent, supra; see also *Elan Pharmaceuticals, 346 F.3d at 1057* (discussing the need for evidence on the question of whether the reference placed a person of ordinary skill in possession of the invention as claimed); *Rosco, Inc. v. Mirror Lite Co., 304 F.3d 1373, 1380 (Fed. Cir. 2002)* ("Under the doctrine of inherency, if an element is not expressly disclosed in a prior art reference, the reference will still be deemed to anticipate [*1372] a subsequent claim if the missing element 'is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill.'") Particularly when the science or technology is new or complex, a bare suggestion or hope that requires significant experimentation for implementation or verification is not an invalidating "anticipation" of that which is ultimately achieved.

These aspects were explored at the trial, with witnesses for both sides agreeing that it was not known, at the time of the Koike reference, how to use cord blood for marrow transplantation and human reconstitution. The district court concluded that a reasonable [**80] jury could have found that no single reference described

all of the *'681 patent* claim limitations, explicitly or inherently. The panel should review this issue in the interest of finality, and rule that the verdict that anticipation of the *'681* claimed invention had not been established was supported by substantial evidence, and was properly sustained by the district court.

The *'553* claims are directed to method steps, including the step of introducing the stem cells into a human host. The district court summarized the evidence as follows:

> It is undisputed that Koike did not introduce cord blood into a human, which is a necessary limitation of the *'553 Patent*. The defendants claim that Koike's suggestion that introducing the stem cells into a human host should be done is a sufficiently enabling disclosure to warrant a finding of anticipation. Even so, the record contains substantial evidence from which a jury could find that a person of ordinary skill in the art would not have been so enabled.

*PharmaStem, 2004 U.S. Dist. LEXIS 18638, 2004 WL 2898061 at *4.*

The defendants argue that Koike is as enabling as the patents in suit--an argument that could well have been rejected by the jury, for the *'681 patent* describes [**81] extensive animal transplantation experiments and shows surrogate assays of over one hundred cord blood units, and the *'553 patent* includes details of the transplantation of cryopreserved fetal cord stem cells to reconstitute the blood of a five-year-old child who was suffering from Fanconi's Anemia; in contrast with the absence of any such information in the Koike reference. The district court held that there was substantial evidence whereby a reasonable jury could have found that the Koike reference did not anticipate the *'553* claims. I agree. The panel should review and resolve this issue, which was fully appealed, in the interest of finality.

Indefiniteness

A similar obligation applies to the cross-appeal of validity on the ground of indefiniteness. The matter was fully presented on the appeal to this court, and warrants resolution.

The defendants challenged both patents under *35 U.S.C. '112*, arguing that the claims are indefinite because, at the time the patent applications were filed, stem cells in umbilical cord and placental blood could not be identified and the stem cell content could not be measured. The defendants' position is that measurement of stem cell content required actually [**82] transplanting the blood into a host and observing its effect, and that since the *'681* composition claims require stem cells "in an amount sufficient to effect hematopoietic reconstitution of a human adult," the defendants could not know if they were infringing the claims. PharmaStem's position is that surrogate animal tests, as shown in its patents, adequately measure stem cell content. PharmaStem points out that the defendants all test the cord blood before placing it in storage and when releasing it for transplant. The jury found [*1373] that the claims were not invalid on this ground, answering Question No. 10:

> Question No. 10
>
> Have the Defendants proven by clear and convincing evidence that the *'681 patent* is indefinite in that on November 12, 1987, a person of ordinary skill in the art would not have been able to determine from the patent what the claimed invention covers?
>
> YES
>
> NO X

Witnesses explained at the trial that the *'681* specification describes the conduct of surrogate assays and their use to test for stem cells, and correlates the surrogate assays with therapeutic stem cell effect. Reviewing the evidence, the district court referred to the expert testimony of Dr. Malcolm Moore, [**83] a cell biologist, that the patents provide "ample information to determine the amount of cord blood needed for transplant in adults and children, and that the scientific community has in fact performed numerous transplants into adults. Moore Tr. at 340-348." *PharmaStem, 2004 U.S. Dist. LEXIS 18638, 2004 WL 2898061 at *5.*

Section 112 requires that the claims point out "the subject matter which the applicant regards as his invention," implementing the purpose of claims to identify what has been invented and found patentable, so that "one skilled in the art would understand the bounds of the claim when read in light of the specification." *Miles Laboratories, Inc. v. Shandon, Inc., 997 F.2d 870, 875 (Fed. Cir. 1993)* ("If the claims read in light of the specification reasonably apprise those skilled in the art of the scope of the invention, *§ 112* demands no more.")

The courts have recognized, particularly in fields of new and evolving knowledge, that the claims can be no more precise than the knowledge in the field permits. See *Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1385 (Fed. Cir. 1986)* ("'if the claims, read in light of the specification, reasonably apprise those skilled in

the art both of the [**84] utilization and scope of the invention, and if the language is as precise as the subject matter permits, the courts can demand no more'") (quoting *Shatterproof Glass Corp. v. Libbey-Owens Ford Co., 758 F.2d 613, 624 (Fed. Cir. 1985))*. See also, e.g., *Marley Mouldings, Ltd. v. Mikron Indus., 417 F.3d 1356, 1361 (Fed. Cir. 2005)* (when a claim "is not insolubly ambiguous, it is not invalid for indefiniteness"); *Exxon Research & Eng'g Co. v. United States, 265 F.3d 1371, 1375 (Fed. Cir. 2001)* ("if the meaning of the claim is discernible, even though the task may be formidable and the conclusion may be one over which reasonable persons will disagree, we have held the claim sufficiently clear to avoid invalidity on indefiniteness grounds"). The defendants argue that even if this criterion is met, it is inadequate to satisfy *'112* in this case because the defendants had no way of being certain whether any unit of cord blood infringed the claims. The defendants argue that even if the science later evolved so that stem cell content could be directly measured, such information did not exist when the *'681* application was filed.

To patent an invention when the science or technology to which it is [**85] directed is incompletely developed or understood, requires that it be described and claimed in terms adequate to communicate, to persons experienced in the field of the invention, what has been discovered. The *'681 patent* states that "any of numerous assays for hematopoietic stem or progenitor cells may be used." Col. 25, lines 49-50. For example:

> [A]n item cell assay for CFU-S (colony forming unit-spleen) can be done. In this assay, cells considered to be multipotential stem cells with self-renewal capacity can be measured by counting the number of colonies (nodules) on the spleen(s) of lethally-irradiated mice that [*1374] have been inoculated with a composition containing the cells.

Col. 26, lines 1-7. The CFU-S assay is done essentially the same way as progenitor cell assays such as BFU-E/CFU-GEMM and CFU-GM assays. Col. 48, lines 42-43. The specification states:

> A survey of published reports indicates that the number of CFU-GM infused for autologous bone marrow reconstitution in human patients, can be relied on as an indicator of the potential for successful hematopoietic reconstitution (Spitzer, G., et al., 1980, Blood 55(2): 317-323; Douay et al., 1986, Exp. Hematol. 14:358-365). By [**86] standardizing published data by patient weight, and assuming a patient weight of 150 pounds (67.5 kilograms), the calculated number of CFU-GM needed for successful hematopoietic reconstitution using autologous bone marrow cells ranges from 2-425x10 <4>, with faster recovery noted using greater than 10x10 <4> CFU-GM.

Col. 50, line 64 to col. 51, line 8. The expert testimony at trial explained this and other descriptive text, whereby a reasonable jury could have concluded that the assays described in the patent serve to ascertain whether sufficient amounts of stem cells are present in the preserved cord blood to reconstitute the host.

It was not disputed that the information in the specification is as definite as the state of scientific knowledge at the time of filing. It has been recognized that the "existence of an inescapable area of uncertainty is not sufficient justification for denying to the patentee the fruits of his invention." *Georgia-Pacific Corp. v. U.S. Plywood Corp., 258 F.2d 124, 136 (2d Cir. 1958)* ("the policy of the patent statute contemplates granting protection to valid inventions, and this policy would be defeated if protection were to be accorded only to those patents which [**87] were capable of precise definition. The judicial function requires a balancing of these competing considerations in the individual case.") The district court fulfilled this judicial function, stating, in denying the defendants' motion for JMOL, that: "Given that there is no determinate or determinable minimum amount of cord blood for therapeutic usefulness in humans, the record supports that the *'681* claim language is as precise as the subject matter permits." *PharmaStem, 2004 U.S. Dist. LEXIS 18638, 2004 WL 2898061 at *5* (citing *Hybritech, supra*). As the district court ruled, there was substantial evidence whereby the jury could have found that the claims of the *'681* and *'553 patents* would be understood by persons in the field of the invention. The verdict that the claims are not invalid for indefiniteness should be sustained, and should be reviewed, not left dangling on appeal.

Obviousness

The ultimate solution of a previously intractable problem can indeed appear to become apparent in hindsight after the final successful step is taken. Yet that final step in this case was not taken by those who came before, and was clearly not "obvious" to contemporaries, who acclaimed the achievement. Even the defendants' expert [**88] witness acknowledged that before the work of these inventors "stem cells could not be conclusively proved to be present in cord blood." Maj. op. at 42. Nonetheless this court rejects the testimony and admissions of the defendants, and uses present knowledge

of the inventors' success to find that it was obvious all along.

When trial is to a jury, the court instructs the jury as to the applicable law, and the jury applies the law to the facts as it finds them. Appellate review is on the standard of determining whether there was substantial evidence to support the jury's express or presumed factual findings, and whether the jury applied the [*1375] correct law to those findings. See *C.R. Bard, Inc. v. M3 Systems, Inc., 157 F.3d 1340, 1351-52 (Fed. Cir. 1998)* ("We review a jury verdict of obviousness to determine whether substantial evidence supports the factual findings predicate to the legal conclusion of obviousness and whether such findings can support the verdict, with appropriate consideration of the presumption of validity and the requirement that obviousness be proved by clear and convincing evidence; factual inferences are drawn and credibility determinations are accepted in favor of the [**89] verdict winner.") The question is whether the jury's verdict is sustainable on the evidence presented, not whether we could have or would have gone the other way on the evidence presented.

The jury answered "NO" to the question whether the *'681* and '553 claimed inventions "would have been obvious to a person of ordinary skill in the field of the invention." Responding to the defendants' challenge to the verdict, PharmaStem points to the evidence of the extensive research in this field of science -- much of which is set forth in the patent specifications -- and to the specific claim limitations. The broadest composition claim (the *'681 patent*) is as follows:

> 1. A cryopreserved therapeutic composition comprising:
>
> > viable human neonatal or fetal hematopoietic stem cells derived from the umbilical cord blood or placental blood of a single human collected at the birth of said human,
> >
> > in which said cells are present in an amount sufficient to effect hematopoietic reconstitution of a human adult;
> >
> > and an amount of cryopreservative sufficient for cryopreservation of said cells.

This claim was the subject of two reexaminations, one preceding this litigation, the second completed during the past year. [**90] For the first reexamination, the examiner's reasons for allowance included the following:

> The claims as amended now avoid the prior art for the following reasons. First, it was noted that the only piece of prior art which taught a composition which could have combined an amount of viable human neonatal or fetal hematopoietic stem cells sufficient to effect hematopoietic reconstitution of a human adult was the reference of Ende. The Ende reference, published in 1972, recited the treatment of an individual undergoing treatment for leukemia who received a series of cord blood infusions from multiple donors and showed a transient change in red blood cell phenotype. Even though the authors of the Ende article describe the procedure as "transplantation," it is clear that such treatment did not result in hematopoietic reconstitution. Further, since no HLA typing was performed, and multiple infusions were performed, one of ordinary skill in the art would have taken the disclosure of Ende to be equivalent to blood transfusions and would have had no expectation that the hematopoietic reconstitution of a human adult could have been performed. As a transfusion composition, one of ordinary skill [**91] would have had no motivation to cryopreserve the cord blood, since whole blood for transfusion is not frozen, but stored st 4 degrees C and Ende further points out that any hospital with a maternity ward would provide sufficient aliquots of fresh cord blood.

Notice of Intent to Issue Reexamination Certificate at 3-4 (Jan. 11, 2000). The examiner discussed the state of the science, the content of the prior art, the known sensitivity of fetal liver and thymus stem cells to freezing, and the unpredictability of this field, and concluded:

> [*1376] This disclosure combined with the acknowledged sensitivity of hematopoietic stem cells from fetal liver and thymus to cryopreservation and the fact that DMSO is toxic to fetal liver progenitor cells at concentrations nontoxic to bone marrow cells provides an unpredictability in the art of cryopreservation of

> stem cells from different sources that renders the suggestions of the prior art references as to the therapeutic uses of umbilical cord blood (whether cryopreserved or not) as a course of hematopoietic stem cells a situation of "obvious to try," which fails to provide a prima facie finding of obviousness . . . .

Id. at 4.

For the second reexamination, [**92] the examiner discussed additional arguments involving the same references on which this court now relies to invalidate the patent:

> At the time of the instant invention the use of cord blood for hematopoietic reconstitution had never been accomplished. Additionally, in vitro expansion of cord-blood stem cells prior to patient implantation had not been successfully employed, and indeed is not in use as of today as indicated by the Dr. Zander declaration. Accordingly, determination of a pharmaceutically efficacious and safe dosage that results in human adult hematopoietic reconstitution would necessarily require undue experimentation, thus precluding enablement. In this respect, it was patentee's in vitro progenitor assays taken in conjunction with in vitro mice testing showing hematopoietic reconstitution with a relatively small amount of neonatal blood, that provided the necessary teaching to enable the obtaining of effective hematopoietic reconstituting dosages in children (extrapolatable to adults) by utilizing cord blood volumes (50-100 ml) derived from a single adult. Thus, neither the Koike reference taken alone anticipates, nor a combination of references render obvious, the instantly [**93] claimed invention.

Reexamination -- Reasons for Patentability/Confirmation (Dec. 29, 2006). No error has been shown in this analysis, which warrants deference in accordance with the strictures of the Administrative Procedure Act. See *Dickinson v. Zurko, 527 U.S. at 164* ("A reviewing court reviews an agency's reasoning to determine whether it is "arbitrary" or "capricious," or, if bound up with a record-based factual conclusion, to determine whether it is supported by "substantial evidence."), citing *SEC v. Chenery Corp., 318 U.S. 80, 89-93, 63 S. Ct. 454, 87 L. Ed. 626 (1943).*

The record contains testimony that scientists working in the field of hematopoietic reconstitution did not expect cord blood to be a successful transplant tissue or a useful source of hematopoietic stem cells. There was testimony that earlier efforts at using cord blood had encountered problems, and that there was skepticism and surprise at the inventors' achievement. The reaction of scientific peers after the achievement is relevant to whether the invention would indeed have been obvious at the time it was made. See *Cardiac Pacemakers, Inc. v. St. Jude Medical, Inc., 381 F.3d 1371, 1376 (Fed. Cir. 2004)* (evidence of skepticism that the multi-mode [**94] treatment of the invention could be achieved supported the jury verdict of nonobviousness); *Metabolite Laboratories, Inc. v. Laboratory Corp. of America Holdings, 370 F.3d 1354, 1368 (Fed. Cir. 2004)* (evidence that skilled artisans were initially skeptical about the invention supported the jury's verdict of nonobviousness).

The significance of the inventors' work was in evidence, including their founding of Biocyte and spawning of the industry of collecting and cryofreezing umbilical cord blood. In evidence was defendant ViaCord's [*1377] "business plan" which identified these inventors as "the trailblazers":

> The founding scientists are core researchers in this field and have published many related articles. Biocyte's time, energies, and financial resources have been spent doing much education and development in this field. They are the trailblazers.

A communication to these inventors from the founder of defendant Cryo-Cell stated:

> [N]o one will ever dispute that you, as the pioneers in the medical technology . . . will be the frontrunners in the field of utilizing of the blood from the umbilical cord for restoring hematopoietic through marrow transplants.

Such evidence assists in replacing [**95] judicial hindsight with objective determination as of the time of the invention. See *Vandenberg v. Dairy Equip. Co., 740 F.2d 1560, 1567 (Fed. Cir. 1984)* (in "determining the question of obviousness, inquiry should always be made into whatever objective evidence of nonobviousness there may be"). In *Graham v. John Deere Co, 383 U.S.*

1, 17-18, 36, 86 S. Ct. 684, 15 L. Ed. 2d 545 (1966) the Court counseled that "Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., "serve to guard against slipping into use of hindsight and to resist the temptation to read into the prior art the teachings of the invention in issue," cited in *KSR v. Teleflex, 127 S. Ct. at 1734.*

PharmaStem's expert, Dr. Bernstein, testified that no prior art showed that cord blood contains stem cells, and that persons of skill in this field would not have had a reasonable expectation of success in carrying out the claimed process. Dr. Bernstein also discussed the early uncertainties and mistaken understanding concerning stem and progenitor cells. His testimony is now disputed by this court, denying it the weight that a reasonable jury could have given it. Dr. Bernstein had explained at the trial that [**96] at the time of filing the patent application the differences between stem cells and progenitor cells could not be measured and were not well understood. The jury could have accepted this testimony, and indeed the defendants did not refute it; but the panel majority now holds that the inventors' apparently inconsistent use of stem and progenitor terminology constitutes an "admission[] in the specification regarding the prior art" which is then "binding on the patentee for purposes of a later inquiry into obviousness." Maj. op. at 42. This is not a simple issue, but the jury could reasonably have concluded, as did the district court, that the prior art did not show that there were stem cells in cord blood, and that one of ordinary skill in this field would not have had a reasonable expectation of successful use of cord blood to reconstitute a human adult.

A reasonable jury could have found that these inventors were not simply conducting a routine optimization, as my colleagues now rule on what they describe as the "more difficult question [of] whether the prior art would have given rise to a reasonable expectation of success in creating the [claimed inventions]." My colleagues state that [**97] they are "plainly satisfied" that "a person of ordinary skill in the art would have had reason to attempt to make [the claimed inventions]." I agree that there was reason to seek a cure for destroyed blood cells, and that scientists have been seeking such a cure for a long time, including those scientists whose work is the cited prior art. There has been much hopeful speculation about the potential of stem cells, although this remedy eluded those who came before.

It is often far easier to recognize the problem than to find and demonstrate the solution. The patent law recognizes that advances of great power may be based as [*1378] much on persistent and skilled investigation as on the flash of creative genius, for both serve to transcend that which was previously achieved. See *35 U.S.C. '103* ("Patentability shall not be negatived by the manner in which the invention was made.") My colleagues go too far in limiting the patent system to the serendipitous and the unexpected. Maj. op. at 35 ("while their work may have significantly advanced the state of the science of hematopoietic transplantations by eliminating any doubt as to the presence of stem cells in cord blood," they "merely used routine [**98] research methods to prove what was already believed to be the case"). Further, these scientists not only established the presence of stem cells, but also enabled their development for preservation and hematopoietic reconstitution.

The court's approach reflects misperception of the scientific process as well as the patent purpose. Scientific methodology usually starts with a hypothesis based on what is already known; the record shows that several scientists mentioned the idea of rebuilding destroyed blood cells. However, none achieved this long-sought goal, and the record shows the extreme skepticism concerning even the possibility of this achievement. The district court found that there was "tremendous skepticism in the transplant field regarding the use of cord blood as a transplant tissue," and that the jury could have found that "prior to the inventions of the Patents-in-suit, those in the field of hematopoietic reconstitution would not have expected cord blood to be a successful transplant tissue."

Nonetheless, my colleagues deny the value of this long-sought result, whereby for the first time umbilical blood was preserved and recovered and used to reconstitute the hematopoietic [**99] systems in mammals, demonstrated with the mice experiments reported in the *'681 patent*, and the human transplant in the *'553 patent*. Not even the defendants denigrate the inventors' achievement as "merely supporting evidence" for an "expected" result, as in the maj. op. at 39. Even if this court were not required to recognize the substantial evidence in support of the jury verdict, even if APA deference were not required to the three PTO reexaminations, one must pause at the powerful evidence of the acclaim that was accorded to this achievement, by these defendants as well as by scientific peers.

There was substantial evidence whereby the jury could have sustained the unobviousness of the *'681* and *'553* inventions. I must, respectfully, dissent from the panel majority's invalidation of these patents on this ground.

INFRINGEMENT

The jury found infringement of the *'681* and *'553 patents*. In determining whether substantial evidence supported the verdict, the evidence before the jury and all reasonable inferences therefrom must be viewed in the light that is favorable to the verdict, without substituting the court's view of the evidence for that of the

jury. *Anderson v. Liberty Lobby, Inc., 477 U.S. 242, 254-55, 106 S. Ct. 2505, 91 L. Ed. 2d 202 (1986)*; [**100] see *SIBIA Neurosciences, Inc. v. Cadus Pharm. Corp., 225 F.3d 1349, 1355 (Fed. Cir. 2000)* ("In reviewing the record, we must draw all reasonable inferences in favor of the prevailing party, and not make credibility determinations or substitute our view of the conflicting evidence for that of the jury.") My colleagues, like the district court, grant JMOL on a ruling of law and evidence that was not presented to the jury, and that in all events does not support reversal of the verdict.

My colleagues appear to hold that infringement cannot be found because the cryopreserved cord blood "relates only as possibilities" for "future use in adult transplants." [*1379] Indeed, this entire system is designed for possible future needs of the infant itself or family members. The defendants' testimony was uniformly to the effect that this "possibility" was the purpose of their preservation service (the record also describes a case in which the cord blood was used to treat the mother's existing disease). The evidence was that most but not all of the cryopreserved cord blood that has been transplanted was to children, with about ten percent transplanted to adults. PharmaStem is correct that this ratio relates [**101] to damages, and does not simply serve to negate all liability for infringement.

The district court ruled that PharmaStem had not proved infringement because PharmaStem did not separately analyze the stem cell content of each sample of cord blood. PharmaStem presented evidence that separate analysis was unnecessary because each defendant had analyzed each sample before accepting it for storage. Every defendant testified that the blood it collected and stored was analyzed for cell content at the time of collection. The jury was not instructed that such evidence was inadequate and inadmissible -- as the district court ruled post-trial. On the evidence presented, this is not a sound basis for rejecting the jury's verdict. The tardy rejection of the testimony of PharmaStem's expert witness, Dr. Hendrix, is an inappropriate application of Daubert and its succeeding cases, on which the panel majority relies, for there was no criticism of the expert's scientific credentials or her analysis of the prior art and the state of the science. See *Daubert v. Merrell Dow Pharms., 509 U.S. 579, 590, 113 S. Ct. 2786, 125 L. Ed. 2d 469 (1993)* (for a scientific assertion to "qualify as 'scientific knowledge,' an inference or assertion must [**102] be derived by the scientific method"); *Kumho Tire Co. v. Carmichael, 526 U.S. 137, 119 S. Ct. 1167, 143 L. Ed. 2d 238 (1999)* (the principles of Daubert apply broadly to "scientific, technical, or other specialized knowledge").

The district court's ground of exclusion was not that Dr. Hendrix made an error of law or of scientific fact, but simply that she also stated her opinion concerning the defendants' marketing statements that they test and preserve cord and neonatal blood for possible future child and adult use -- testimony that the district court criticized because it did not require scientific expertise. Whatever the virtue of that criticism, it is clear that the district court's (and my colleagues') exclusion of the entire testimony of this eminent scientist on this ground is not what the Daubert ruling is about. There was no testimony contrary to the view of Dr. Hendrix of the scope of the representations made in the marketing materials, and no challenge to the accuracy of her statements. Presentation of expert testimony was in compliance with the general rule that "typically expert testimony will be necessary in cases involving complex technology," *Centricut, LLC v. Esab Group, Inc., 390 F.3d 1361, 1370 (Fed. Cir. 2004)*, [**103] and this expert's testimony did not cross the boundaries of admissibility.

The '681 Patent

The district court granted JMOL of noninfringement of the *'681 patent* on the ground that PharmaStem had not proved that 100% of the defendants' preserved cord and neonatal blood contained sufficient stem cells to reconstitute an adult. The district court reasoned that since PharmaStem took the litigation position that it was entitled to damages measured as a royalty based on 100% of the preserved blood, to prove infringement PharmaStem had to prove that 100% of the preserved blood contained sufficient stem cells to provide adult reconstitution, by analyzing 100% of the preserved blood. As I have mentioned, PharmaStem complains that this criterion differed from that [*1380] on which the jury was instructed, and also states that even this criterion was met by substantial evidence presented at the trial.

My colleagues, overturning the jury verdict, hold that there is no infringement of the *'681 patent* because PharmaStem did not retest every unit of stored blood to determine its stem cell content. They ignore the evidence that every unit was tested by each defendant before being placed into cryogenic storage; [**104] every defendant so testified. It was not disputed that retesting of every unit could use up a significant amount of the precious preserved blood. No defendant asserted that it routinely cryogenically preserved cord blood that did not contain sufficient stem cells to be potentially useful for hematopoetic reconstitution. A reasonable jury could have considered this evidence to find that each element of the claims was met. Instead, my colleagues simply rule that without testing of the stored units there can be no liability at all. That evidentiary theory was not presented to the jury; it is too late to criticize as legally inadequate the testimony that was based on the defendants' own representations concerning the content of the stored umbilical and neonatal blood.

The verdict of infringement was supported by the defendants' own testimony setting forth their requirements for stem cell content before accepting cord blood for cryopreservation. For example, defendant CBR's Scientific Director testified that every unit of cord blood presented to CBR for storage is tested to see if it contains a sufficient amount of stem cells to have "a [**105] good probability of being useful in the clinical setting." In evidence were CBR's website statements that "transplants have occurred for the newborn himself, the newborn's mother, father, and the newborn's cousin," and "umbilical cord blood from unrelated donors can restore hematopoiesis in adults who receive myeloablative therapy and associated with acceptable rates of severe acute and chronic GVHD [Graft vs. Host Disease]."

The President of defendant CorCell testified that "what our marketing materials state [is] that it may be used to treat the donor or siblings or potentially parents," and that although only one CorCell stored cord blood unit had thus far been transplanted, that transplant was to an adult. There was testimony that CorCell's cord blood samples are tested for "total nucleated, CD-34+ and viability cell counts before and after processing," and "a colony-forming assay is conducted to evaluate the quality and quantity of umbilical stem cells," and that a sample is usually not preserved if its stem cell content is determined to be unsuitable for possible future use. The jury was shown CorCell's representation to investors that "a recent study of twenty-five (25) patients, [**106] published in the New England Journal of Medicine, similarly indicates that cryopreserved umbilical cord blood stem cells can be successfully engrafted in children and adults with a variety of hematologic or immunologic disorders." The jury saw evidence that CorCell defines potential recipients of the stored stem cells as "the family members of the newborn, mother, father, siblings and possibly grandparents."

Defendant ViaCell's founder testified that each cord blood sample was tested to ensure that there is a sufficient amount of stem cell content to be therapeutically useful, as determined by ViaCell's Scientific Advisory Board. ViaCell's Senior Vice President testified that ViaCell counts the cells in every collected sample, and that its standard procedure states: "A minimum total NC count of 3.0 x 10 <8> is required to proceed with processing." A ViaCell memorandum to investors stated that about 10% of all cord blood transplants were in adults, and a ViaCell witness testified [*1381] that ViaCell informs the public about adult use.

At the trial none of the defendants denied the stem cell content of the blood they cryopreserved, other than to state that for the few cases where their analysis [**107] at collection showed weak stem cell content they would consult with the infant's family before accepting and freezing the blood. The jury heard the defendants' testimony and unqualified representations concerning their screening of every stored sample of cord blood for stem cell content, and that they did not distinguish between potential child and adult use of the stem cells. The jury could have relied on the defendants' testimony that their minimum threshold for cryopreservation is sufficient stem cells for transplantation, and that all of the defendants included possible adult use in their publicly-stated reasons for storing fetal cord and neonatal blood. PharmaStem points out that it was neither necessary nor prudent to test each unit of the defendants' stored blood for stem cell content, when each defendant had already done so.

The jury was instructed: "A defendant is liable for directly infringing PharmaStem's patents if you find that PharmaStem has proven by a preponderance of the evidence that they have made, used, offered for sale or sold a composition that includes each and every element of at least one of the asserted claims of the *'681 patent*." The theory that each stored [**108] sample had to be separately analyzed by PharmaStem to show infringement was not presented as law to the jury. This was a new standard for infringement, for the jury was not told that the defendants' analyses of stem cell content could not provide evidence of stem cell content.

When there is substantial evidence in support of the jury's verdict, it is irrelevant whether the appellate court would have preferred different or additional evidence. "When the jury is supplied with sufficient valid factual information to support the verdict it reaches, that is the end of a matter . . . . the jury's factual conclusion may not be set aside by a JMOL order." *McGinley v. Franklin Sports, Inc., 262 F.3d 1339, 1355 (Fed. Cir. 2001)*. The district court erred in holding that it was necessary for PharmaStem to analyze, or provide detailed analysis results, for the individual blood units in order to find infringement. My colleagues commit the same error, reweighing the evidence to reach their preferred result, rather than considering whether substantial evidence as presented at the trial supports the verdict that was reached by the jury.

The '553 Patent

It was agreed at trial that the claims of the *'553 patent* [**109] are not infringed until the step of transplanting the stem cells takes place. Since relatively few transplants of stored blood had been done, the royalties awarded by the jury were modest, and were not appealed. However, the verdict of infringement is supported by substantial evidence, and should stand. There was substantial evidence that each step of the claimed invention is performed by the defendants followed by a transplant surgeon. Referring to claim 13, the defendants isolate the

umbilical cord and placental blood containing stem cells and cryopreserve it in liquid nitrogen; claim clauses (a) and (b). When instructed on behalf of the donor or family members, the blood is delivered to a surgical environment where it is thawed, claim clause (c), and transplanted into the human host, claim clause (d):

> 13. A method for hematopoietic or immune reconstitution of a human comprising:
>
> (a) isolating human neonatal or fetal blood components containing hematopoietic stem cells;
>
> [*1382] (b) cryopreserving the blood components;
>
> (c) thawing the blood components; and
>
> (d) introducing the blood components into a suitable human host, such that the hematopoietic stem cells are viable and can proliferate within [**110] the host.

The jury found the defendants liable for "acting in concert or working together" with the transplant physicians, or contributing to the infringement of the *'553 patent*, upon answering the following questions:

> Question No. 3: Substantial Non-Infringing Use
>
> Has PharmaStem proven by a preponderance of the evidence that cryopreserved cord blood has no substantial noninfringing use?
>
> YES X NO
>
> Question No. 4: Direct Infringement
>
> Has PharmaStem proven by a preponderance of the evidence that defendants and the transplant physicians are acting in concert or working together to complete the process of infringement of claims 13, 19, 47, 53, or 57 of the *'553 patent* by performing each and every one of the steps in any of those claims?
>
> YES X NO
>
> Question No. 5: Contributory Infringement
>
> Has PharmaStem proven that a defendant has contributorily infringed the *'553 patent* by selling or offering to sell cryopreserved cord blood that was actually used by a third party in the direct infringement of any of claims 13, 19, 47, 53, or 57 of the *'553 patent*?
>
> Answer separately for each defendant.
>
> ViaCell YES X NO
>
> CBR YES X NO
>
> Cryo-Cell YES X NO
>
> CorCell YES X NO

PharmaStem thus received special [**111] verdicts of both direct joint infringement and contributory infringement. My colleagues grant JMOL on the ground that since the defendants are providing a service, not selling a product, they can not meet the "sale" requirement of con-

tributory infringement, *35 U.S.C. § 271(c)*. [1] PharmaStem points out that a reasonable jury could have found that the defendants sell (rent) their blood-storage facilities to the donor's family, and that the defendants either contribute to or act in concert with the transplanting surgeon to practice the claimed method.

> 1 *§ 271(c)*. Whoever offers to sell or sells within the United States or imports into the United States a component of a patented machine, manufacture, combination or composition, or a material or apparatus for use in practicing a patented process, constituting a material part of the invention, knowing the same to be especially made or especially adapted for use in an infringement of such patent, and not a staple article or commodity of commerce suitable for substantial noninfringing use, shall be liable as a contributory infringer.

The principles of patent infringement are not negated when the steps of a method claim are performed by more than [**112] one entity. There was no instruction as to legal impossibility of liability as to the *'553 patent*, and no objection was raised to the verdict questions. We are not told whether the legal theory of sale or rent was aired at the trial, but it is apparent that the jury was fully apprised of the nature of the accused activities, as reflected in the jury questions. The processes of litigation require appellate review on the premises of the jury trial, lest invited error dominate trial tactics.

No objection was raised to the jury instructions. The distinction relied on by [*1383] the panel majority, that the defendants were bailees, not sellers, does not negate the principles of infringement, whether viewed as joint infringement or contributory infringement. See, e.g., *On Demand Machine Corp. v. Ingram Indus., Inc., 442 F.3d 1331, 1334 (Fed. Cir. 2006)* (approving instruction that "It is not necessary for the acts that constitute infringement to be performed by one person or entity.") PharmaStem is correct that the issue to which this evidence applies relates to damages, not infringement, and points to the small amount of damages awarded for infringement of the *'553 patent* (damages for the *'553 patent* [**113] were not appealed by the defendants).

It is irrelevant whether any steps of a method claim can be viewed as a "service;" infringement requires only that the steps be performed. As discussed in *Dawson Chemical Co. v. Rohm and Haas Co., 448 U.S. 176, 188, 100 S. Ct. 2601, 65 L. Ed. 2d 696 (1980)*, the purpose of the contributory infringement statute is "to protect patent rights from subversion by those who, without directly infringing the patent themselves, engage in acts designed to facilitate infringement by others," a criterion that the jury could have found was met by the facts and relationships of this case. On the instructions to the jury, the verdict of liability for contributory or joint infringement of the *'553 patent* is supported by substantial evidence, and should be sustained.

From the court's departure from the procedures of appellate review of jury verdicts, and from the flawed law that is propounded, I must, respectfully, dissent.

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LEXSEE 437 F.3D 1157

MEDICHEM, S.A., Plaintiff-Appellee, v. ROLABO, S.L., Defendant-Appellant.

05-1179, 05-1248

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

437 F.3d 1157; 2006 U.S. App. LEXIS 2653; 77 U.S.P.Q.2D (BNA) 1865

February 3, 2006, Decided

SUBSEQUENT HISTORY: Rehearing denied by, Rehearing, en banc, denied by *Medichem, S.A. v. Rolabo, S.L., 2006 U.S. App. LEXIS 7669 (Fed. Cir., Mar. 15, 2006)*

PRIOR HISTORY: [**1] Appealed from: United States District Court for the Southern District of New York. Judge Jed S. Rakoff.
Medichem, S.A. v. Rolabo, S.L., 2004 U.S. Dist. LEXIS 23697 (S.D.N.Y., Nov. 19, 2004)

DISPOSITION: AFFIRMED-IN-PART, REVERSED-IN-PART.

CASE SUMMARY:

PROCEDURAL POSTURE: In an action for patent interference under *35 U.S.C.S. § 291*, defendant manufacturer appealed from a judgment on remand for the second time, in which the United States District Court for the Southern District of New York found the existence of an interference-in-fact and awarded priority of invention to plaintiff manufacturer.

OVERVIEW: The case dealt with the active pharmaceutical ingredient in a popular allergy medication. The only significant difference between the parties' processes was that plaintiff's process required the presence of a chemical known as tertiary amine. Defendant argued that the district court erred in finding the existence of an interference-in-fact based on its finding that plaintiff's invention would have been obvious over the broader invention by defendant. The appellate court found no error, concluding that skilled artisans in possession of defendant's patent would have not only been motivated to add the chemical, but would have possessed a reasonable expectation that they would succeed in optimizing the reaction by adding it. Defendant also argued that the district court erred in awarding priority of invention to plaintiff based on the oral testimony of co-inventor, which defendant claimed was not corroborated by independent evidence. The appellate court agreed, concluding that the modicum of additional corroborative value that could be assigned to a non-inventor's notebook fell short of that necessary for the notebook to be used to corroborate the alleged reduction to practice date.

OUTCOME: The district court's determining that an interference-in-fact existed was affirmed. The district court's award of priority to plaintiff was reversed.

LexisNexis(R) Headnotes

Patent Law > U.S. Patent & Trademark Office Proceedings > Interferences > Interference in Fact
[HN1] The first step in an interference analysis is for a court to determine whether an interference exists under *35 U.S.C.S. § 291* by asking whether the patents have the same or substantially the same subject matter in similar form as that required by the PTO pursuant to *35 U.S.C.S. § 135*. In order to make this determination, the court uses a "two-way" test which states that two patents interfere only if (1) invention A either anticipates or renders obvious invention B, where Party A 's claimed invention is presumed to be prior art vis-a-vis Party B and (2) vice versa.

Civil Procedure > Appeals > Standards of Review > General Overview
Patent Law > U.S. Patent & Trademark Office Proceedings > Interferences > Interference in Fact
[HN2] In reviewing a district court's finding of an interference-in-fact pursuant to the two-way test, an appellate court reviews, where necessary, both the subsidiary findings of anticipation and/or obviousness as they relate to the application of the test.

Civil Procedure > Appeals > Standards of Review > Clearly Erroneous Review
Civil Procedure > Appeals > Standards of Review > De Novo Review
Patent Law > Nonobviousness > General Overview
Patent Law > Nonobviousness > Evidence & Procedure > Fact & Law Issues
[HN3] In a patent context, obviousness under *35 U.S.C.S. § 103* is a legal conclusion that is reviewed de novo; however, it is based in turn on underlying factual determinations which are reviewed for clear error. Under the clear error standard, a reversal is permitted only when an appellate court is left with a definite and firm conviction that the district court was in error.

Patent Law > Nonobviousness > Evidence & Procedure > Fact & Law Issues
[HN4] The ultimate determination of whether an invention would have been obvious under *35 U.S.C.S. § 103(a)* is a legal conclusion based on the factual Graham findings, e.g., (1) the scope and content of the prior art; (2) the level of ordinary skill in the prior art; and (3) the differences between the claimed invention and the prior art.

Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN5] If all the elements of an invention are found in a combination of prior art references, a proper analysis under *35 U.S.C.S. § 103* requires, inter alia, consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and (2) whether the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have a reasonable expectation of success.

Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
Patent Law > Nonobviousness > Evidence & Procedure > Fact & Law Issues
[HN6] In making obviousness determinations, the test is whether the subject matter of the claimed inventions would have been obvious to one skilled in the art at the time the inventions were made, not what would be obvious to a judge after reading the patents in suit and hearing the testimony. Whether such a motivation has been demonstrated is a question of fact. Evidence of a motivation to combine prior art references may flow from the prior art references themselves, the knowledge of one of ordinary skill in the art, or, in some cases, from the nature of the problem to be solved.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
Patent Law > Nonobviousness > Elements & Tests > Teaching Away From Invention
[HN7] When a piece of prior art suggests that the line of development flowing from the reference's disclosure is unlikely to be productive of the result sought by the applicant, the piece of prior art is said to "teach away" from the claimed invention. As with other subsidiary obviousness inquiries, what a reference teaches and whether it teaches toward or away from the claimed invention are questions of fact. However, obviousness must be determined in light of all the facts, and there is no rule that a single reference that teaches away will mandate a finding of nonobviousness. Likewise, a given course of action often has simultaneous advantages and disadvantages, and this does not necessarily obviate motivation to combine. Where the prior art contains "apparently conflicting" teachings (i.e., where some references teach the combination and others teach away from it) each reference must be considered for its power to suggest solutions to an artisan of ordinary skill, considering the degree to which one reference might accurately discredit another.

Patent Law > Nonobviousness > General Overview
[HN8] Obviousness does not require absolute predictability of success. All that is required is a reasonable expectation of success.

Patent Law > Nonobviousness > General Overview
[HN9] To have a reasonable expectation of success, one must be motivated to do more than merely to vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful. Similarly, prior art fails to provide the requisite "reasonable expectation" of success where it teaches merely to pursue a general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it.

Civil Procedure > Appeals > Standards of Review > Clearly Erroneous Review
Patent Law > Nonobviousness > Evidence & Procedure > Fact & Law Issues
[HN10] A district court's finding of a reasonable expectation of success is a question of fact, which an appellate court reviews for clear error.

Patent Law > Date of Invention & Priority > General Overview
Patent Law > U.S. Patent & Trademark Office Proceedings > Interferences > General Overview
[HN11] At least a single description of an interfering subject matter is necessary for a determination of priority.

Patent Law > Date of Invention & Priority > General Overview
[HN12] Under *35 U.S.C.S. § 291*, a party that does not have the earliest effective filing date needs only to demonstrate by a preponderance of the evidence that it was the first to invent if the two patents or applications at issue were co-pending before the PTO.

Patent Law > Date of Invention & Priority > Conception Date
Patent Law > Date of Invention & Priority > Reduction to Practice
[HN13] Priority of invention goes to the first party to reduce an invention to practice unless the other party can show that it was the first to conceive of the invention and that it exercised reasonable diligence in later reducing that invention to practice.

Patent Law > Date of Invention & Priority > Reduction to Practice
[HN14] Where neither party relied on a date of conception, priority of patent is properly awarded to the party that was the first to reduce its invention to practice, either actually or constructively.

Patent Law > Date of Invention & Priority > Reduction to Practice
[HN15] In order to establish an actual reduction to practice, a party must establish three things: (1) construction of an embodiment or performance of a process that met all the limitations of the interference count; (2) determination that the invention would work for its intended purpose, and (3) the existence of sufficient evidence to corroborate inventor testimony regarding these events.

Patent Law > Date of Invention & Priority > Corroboration of Invention Date
Patent Law > Date of Invention & Priority > Reduction to Practice
[HN16] No condition of "corroboration" is imposed on an inventor's notebook, or indeed on any documentary or physical evidence, as a condition for its serving as evidence of reduction to practice. However, in a case involving reduction to practice, an unwitnessed notebook is insufficient on its own to support a claim of reduction to practice.

Patent Law > Date of Invention & Priority > Corroboration of Invention Date
[HN17] Sufficiency of corroboration is determined by using a "rule of reason" analysis, under which all pertinent evidence is examined when determining the credibility of an inventor's testimony.

Patent Law > Date of Invention & Priority > Corroboration of Invention Date
[HN18] The requirement of independent knowledge remains key to the corroboration inquiry. Independent corroboration may consist of testimony of a witness, other than the inventor, to the actual reduction to practice or it may consist of evidence of surrounding facts and circumstances independent of information received from the inventor. One consequence of the independence requirement is that testimony of one co-inventor cannot be used to help corroborate the testimony of another.

Patent Law > Date of Invention & Priority > Corroboration of Invention Date
[HN19] The law does not impose an impossible standard of "independence" on corroborative evidence by requiring that every point of a reduction to practice be corroborated by evidence having a source totally independent of the inventor. Similarly, it is not necessary to produce an actual over-the-shoulder observer. Rather, sufficient circumstantial evidence of an independent nature can satisfy the corroboration requirement.

Patent Law > Date of Invention & Priority > Corroboration of Invention Date
Patent Law > Date of Invention & Priority > Reduction to Practice
[HN20] When an inventor claims a process for making a chemical compound rather than the compound itself, it is the successful reduction to practice of the process that

must be corroborated, and not merely the successful production of the compound per se. Thus, spectral evidence that might be sufficient per se to corroborate a claim directed to the product will generally not be sufficient to corroborate a claim directed to the process, in the absence of some evidence to corroborate that the product was produced via that process.

Civil Procedure > Appeals > Standards of Review > Clearly Erroneous Review
Patent Law > Date of Invention & Priority > Corroboration of Invention Date
[HN21] Whether or not corroboration exists is a question of fact, the district court's determination of which an appellate court reviews for clear error.

Civil Procedure > Appeals > Standards of Review > Clearly Erroneous Review
Patent Law > Date of Invention & Priority > Corroboration of Invention Date
[HN22] Corroboration is fundamentally about "credibility," and in reviewing factual findings under the clear error standard, an appellate court gives great deference to a district court's decisions regarding credibility of witnesses.

Civil Procedure > Appeals > Standards of Review > General Overview
Civil Procedure > Appeals > Standards of Review > Clearly Erroneous Review
Patent Law > Date of Invention & Priority > Corroboration of Invention Date
[HN23] Even the most credible inventor testimony is a fortiori required to be corroborated by independent evidence, which may consist of documentary evidence as well as the testimony of non-inventors. To the extent that a district court's finding of corroboration rests on its assessment of the credibility of non-inventor testimony, an appellate court applies a deferential standard of review. To the extent that it rests on the district court's assessment of documentary, as opposed to testimonial evidence, an appellate court still applies clear error review; however, clear error is less difficult to establish.

Patent Law > Date of Invention & Priority > Corroboration of Invention Date
[HN24] Where a laboratory notebook authored by a non-inventor is offered into evidence pursuant to authentication by an inventor, where the author of the notebook has not testified at trial or otherwise attested to its authenticity, and where the notebook has not been signed or witnessed and has not been maintained in reasonable accordance with good laboratory practices sufficient to reasonably ensure its genuineness under the circumstances, then the corroborative value of the notebook is minimal.

COUNSEL: John G. Taylor, Frommer Lawrence & Haug LLP, of New York, New York, argued for plaintiff-appellee. With him on the brief were Barry S. White and James K. Stronski.

Thomas P. Heneghan, Michael Best & Friedrich LLP, of Madison, Wisconsin, argued for defendant-appellant. With him on the brief were Jeffrey S. Ward and Charlene L. Yager.

JUDGES: Before SCHALL, GAJARSA, DYK, Circuit Judges.

OPINION BY: GAJARSA

OPINION

[*1160] GAJARSA, Circuit Judge.

This is the second round of a protracted litigation to establish priority of invention between Stampa et al.'s *U.S. Patent No. 6,084,100* ("the *'100 patent*"), assigned to Medichem, S.A. ("Medichem"), and Jackson's *U.S. Patent No. 6,093,827* ("the *'827 patent*"), assigned to Rolabo, S.L. ("Rolabo"). In the first round appealed to this court, we remanded to the district court, requiring it to establish an interference-in-fact under *35 U.S.C. § 291* before determining priority. *Medichem, S.A. v. Rolabo, S.L., 353 F.3d 928 (Fed. Cir. 2003)* ("Medichem II"). Rolabo now appeals [**2] from the judgment on remand, in which the United States District Court for the Southern District of New York found the existence of an interference-in-fact and awarded priority of invention to Medichem. See *Medichem, S.A. v. Rolabo, S.L., Memorandum Order, 2004 U.S. Dist. LEXIS 23697, No. 01 Civ. 3087, 2004 WL 2674632 (S.D.N.Y Nov. 22, 2004)* ("Medichem III"). For the reasons discussed below, we affirm the judgment of the district court on the proper establishment of the interfering subject matter and on the finding of the existence of an interference-in-fact. We reverse, however, the district court's award of priority to Medichem, based on the insufficiency of the evidence that Medichem introduced at trial to corroborate the testimony of its inventors regarding reduction to practice of the invention.

BACKGROUND

A. The Patents

Medichem and Rolabo are both pharmaceutical manufacturers based in Barcelona, Spain. Rolabo's *'827*

patent and Medichem's *'100 patent* both claim a process for making loratadine from two precursor chemicals via a chemical reaction known as the McMurry reaction. Loratadine is the active ingredient in the allergy medication Claritin (R). McMurry reactions involve the [**3] coupling of two starting materials in the presence of low-valent titanium. In general, McMurry reactions can lead to two types of products, diols and alkenes; loratadine, the desired end product of this reaction, is an alkene. McMurry reactions can be optimized for alkene production by adjusting various reaction parameters, such as the temperature and length of the reaction in this case, and also by adding additional reactants. The only significant difference between the processes claimed by Medichem [1] and Rolabo [2] is that Medichem's [*1161] process requires the reaction to be carried out in the presence of a type of chemical known as a tertiary amine. [3] In contrast, the Rolabo process permits by not excluding, but does not require, the presence of a tertiary amine. Conceptually, therefore, the Medichem invention, which requires a tertiary amine, is a species within the genus of the Rolabo invention.

1 Claims 1 and 2 of Medichem's *'100 patent* read:

> 1. A process for the preparation of loratadine consisting of reacting, in an organic solvent and in the presence of a tertiary amine, 8-chloro-5,6- dihyro-benzo[5,6]cyclohepta[1,2-b]pyridin-11-one, of formula VII with a low-valent titanium species. (emphasis added).
>
> 2. The process of claim 1, wherein the low-valent titanium species are generated by reduction of titanium tetrachloride with zinc dust.

[**4]

2 Claims 1 and 17 of Rolabo's *'827 patent* read:

> 1. A process for preparing 5,6-dihydro-11H-dibenzo[a,d]cyclohept-11-enes comprising reacting a dibenzo-suberone or an aza derivative thereof with an aliphatic ketone in the presence of low valent titanium wherein said low valent titanium is generated by zinc.
>
> 17. A process as claimed in claim 1 for preparing Loratadine.

3 A tertiary amine is a compound in which nitrogen is bonded three times to carbon. A commonly used tertiary amine is pyridine.

B. Proceedings to Date

Medichem brought an action under *35 U.S.C. § 291*, alleging an interference-in-fact between the *'100* and *'827 patents*, claiming priority of invention, and seeking invalidation of Rolabo's patent under *35 U.S.C. § 102(g)*. Transcript of Verdict at 653-67, *Medichem, S.A. v. Rolabo, S.L., No. 01 Civ. 03087, 2002 U.S. Dist. LEXIS 27086 (S.D.N.Y May 8, 2002)* ("Medichem I"). Because Rolabo was the party with the earlier effective filing date, Medichem sought to establish priority by proving an actual reduction to practice that was even earlier. [4] After a bench trial, the district [**5] court found that there was no interference-in-fact between the claimed inventions, but it nonetheless awarded priority to Medichem. Id.

4 Rolabo's effective filing date is February 26, 1997 and Medichem's is May 30, 1997.

On appeal, this court vacated the priority holding, opining that because the existence of an interference-in-fact is a jurisdictional requirement under *35 U.S.C. § 291*, it was therefore a precondition to the district court's consideration of the priority issue. *Medichem II, 353 F.3d at 935-36*. We explained that [HN1] the first step in an interference analysis is for the court to determine whether an interference exists under *35 U.S.C. § 291* by asking whether the "patents. . . have the same or substantially the same subject matter in similar form as that required by the PTO pursuant to *35 U.S.C. § 135*." *Id. at 934* (internal quotations omitted). In order to make this determination, we use the "two-way" test which states that two patents interfere only if (1) invention *A* either anticipates or renders obvious invention *B*, where Party *A* [**6] 's claimed invention is presumed to be prior art vis-a-vis Party *B* and (2) vice versa. Id. (citing *Eli Lilly & Co. v. Bd. of Regents of the Univ. of Wash., 334 F.3d 1264, 1268 (Fed. Cir. 2003))*.

In Medichem II, we held that Medichem's claims to the "species" would clearly anticipate Rolabo's genus claim if the Medichem patent were assumed to be prior art. *Id. at 934-35*. Thus, we held that the first prong of the two-way test was clearly satisfied. *Id. at 935*. However, we remanded to the district court for a determination of whether the second prong was also satisfied-namely, whether Rolabo's [*1162] genus claim, if prior

art, would either anticipate or render obvious Medichem's species claim. *Id. at 935*. We explained that "as the *'827 patent* contains genus claims and the *'100 patent* contains species claims, an arrangement that assumes that the *'827 patent* is prior art does not necessarily anticipate or make obvious the narrower claims of the *'100 patent*." Id.

On remand, the district court held that "assuming arguendo [pursuant to the two-way test] the priority of the *'827 patent*, claims 1 and 17 of the *'827 patent* clearly anticipate and render [**7] obvious the adding of a tertiary amine, as in the *'100 patent*." *Medichem III, 2004 U.S. Dist. LEXIS 23697, 2004 WL 2674632 at *7*. Although the court went on to explain its holding on obviousness grounds, it was silent about the reasons underlying its apparent determination that Rolabo's genus claims would also anticipate Medichem's species claim. Instead, it improperly recharacterized our remand instructions as "reducing to the question of whether it would be 'obvious' to add tertiary amine to a McMurry reaction to make loratadine." [5] Id.(emphasis added).

> 5 In so doing, the court appears not to have separately considered the question of whether the *'827 patent*, if taken as prior art, would anticipate the *'100 patent*.

The court then correctly stated that:

> Determining obviousness requires consideration of two factors: 1) whether the prior art would have suggested to one of ordinary skill in the art that he should carry out the claimed process; and 2) whether the prior art would have also revealed that in carrying out the process, one of ordinary skill would have a reasonable expectation of success.

Id. The district court proceeded to articulate [**8] factual bases for its obviousness holding, which included (1) an article that pointed to the use of amines to improve yields in coupling reactions, (2) testimony by Rolabo's expert about additional such prior art, and (3) evidence that such prior art had actually motivated Medichem's inventor's to try adding tertiary amine to the reaction mixture. *Medichem III, 2004 U.S. Dist. LEXIS 23697, 2004 WL 2674632 at *7-8*.

Having found the two-way test's second prong to be satisfied on both anticipation and obviousness grounds, the district court concluded that the Medichem and Rolabo patents interfered, a finding that gave it jurisdiction over the priority dispute pursuant to *35 U.S.C. § 291*. It awarded priority to Medichem, after finding that the invention claimed in the *'100 patent* was reduced to practice prior to the constructive reduction to practice date of Rolabo's invention. See *2004 U.S. Dist. LEXIS 23697, [WL] at *10-11* (referring to Medichem I and stating that the court "reinstates and reaffirms its former priority ruling").

In finding reduction to practice, the court neither explicitly discussed the legal requirement that reduction to practice be corroborated by independent evidence, [**9] nor made a factual finding of corroboration. However, it dismissed Rolabo's argument that Medichem's inventors were not credible as a result of having fraudulently backdated documents that it had offered to show reduction to practice in 1995. The court thus affirmed its finding in Medichem I that Medichem had provided adequate proof of reduction to practice in 1996. The court did so notwithstanding its previous observation that "the willingness of Medichem to fraudulently backdate [evidence of reduction to practice in 1995], coupled with Medichem's less than punctilious recordkeeping practices . . . does convince the Court that it cannot place the same reliance on plaintiff's testimony and documents as it might otherwise have." Transcript of Verdict at 658, MedichemI. However, the court apparently adhered to [*1163] its view that Medichem's fraudulent backdating was "chiefly a belated attempt to deal with their noncompliance with [certain] regulatory requirements." Id. The Medichem III court therefore reaffirmed its award of priority to Medichem, and Rolabo appealed on February 9, 2005. This court has jurisdiction pursuant to *28 U.S.C. § 1295* [**10] *(a)(1)*.

As an aside, we wish to note that in parallel with the district court proceedings under *35 U.S.C. § 291*, the Board of Patent Appeals and Interferences ("Board") has been considering essentially the same interference and priority issues pursuant to *35 U.S.C. § 135*. See *Stampa v. Jackson, 2002 Pat. App. LEXIS 191, 65 U.S.P.Q.2d 1942 (B.P.A.I. 2002)* (involving an interference between Medichem's then-pending reissue application and both Rolabo's patent and a pending continuation application thereof, giving rise to Patent Interference Nos. 105,069 and 105,212). The Board held that the district court's holding in Medichem I did not bar the Board proceedings on grounds of issue preclusion. See *id. at 1945-47*.

Shortly after the district court's remand decision in Medichem III, the Board resolved the interference in favor of Rolabo, reaching a conclusion opposite to that of the district court. See *Stampa v. Jackson, 76 U.S.P.Q.2d (BNA) 1105, Inter. Nos. 105,069 & 105,212, 2005 Pat. App. LEXIS 12, 2005 WL 596770 (B.P.A.I. January 25, 2005)*. Central to its decision was Medichem's failure to corroborate its account of an alleged actual reduction [**11] to practice with evidence independent of its inventors' testimony. *76 U.S.P.Q.2d (BNA)*

*1105, 2005 Pat. App. LEXIS 12, [WL] at *19-20.* The Board noted that "all of the evidence regarding an experiment on May 7, 1996 which is said to have obtained loratadine via a process of the count and conducted by [non-inventor] Lola Casas and said to be recorded [in her notebook] is based on the testimony of [Medichem inventors]." *76 U.S.P.Q.2d (BNA) 1105, 2005 Pat. App. LEXIS 12, [WL] at *15.* Significantly, Medichem did not produce any testimony from Casas, a failure that the Board perceived as sufficient to permit the inference that Casas' testimony would have been adverse to Medichem. *76 U.S.P.Q.2d (BNA) 1105, 2005 Pat. App. LEXIS 12, [WL] at *20.* However, the Board declined to apply such an adverse inference on the grounds that "[Medichem's] case is so weak, we find it unnecessary to draw an inference one way or the other." [6] Id. While appellant does not argue that the Board decision as a binding effect on this court, Board decisions nevertheless represent the views of a panel of specialists in the area of patent law. Medichem has appealed the Board's decision to this court. See Stampa v. Jackson, appeal docketed, Nos. 06-1004 & -1029 (Fed. Cir. Oct. 6, 2004 & Oct. 24, 2004).

> 6 A final judgment on the merits was issued the same day. See *Stampa v. Jackson, 76 U.S.P.Q.2d (BNA) 1105, Inter. Nos. 105,069 & 105,212, 2005 Pat. App. LEXIS 12, 2005 WL 596770 (B.P.A.I. January 25, 2005).* The Board later denied Medichem's request for rehearing, stating inter alia that "the importance of Lola Casas' testimony is manifest. She is the principal, if not the only, corroborating witness on the issue of whether an actual reduction to practice took place." See *Stampa v. Jackson, Inter. Nos. 105,069 & 105,212, 2006 Pat. App. LEXIS 40, 2005 WL 1541082 (B.P.A.I. June 27, 2005).*

[**12] DISCUSSION

There are three issues in this case-namely, whether the district court (1) erred in finding the existence of an interference-in-fact; (2) committed reversible error in failing to formally define a count corresponding to the interfering subject matter; and (3) erred in awarding priority of invention to Medichem based on the oral testimony of Medichem co-inventors, testimony that Rolabo claims was not corroborated by independent evidence, and thus should not have been credited in the final determination of whether reduction to practice was established before the critical date.

[*1164] A. Existence of an Interference-in-Fact

For the reasons explained below, we agree that under the second prong of the two-way test for obviousness, Rolabo's genus claim renders obvious the Medichem species claim. We therefore affirm the lower court's finding of an interference-in-fact without needing to review the district court's unsupported factual finding that the second prong of the two-way test was independently satisfied on anticipation grounds.

1. Standard of Review

[HN2] In reviewing a district court's finding of an interference-in-fact pursuant to the two-way test, this court reviews, where [**13] necessary, both the subsidiary findings of anticipation and/or obviousness as they relate to the application of the test. See *Medichem II, 353 F.3d at 932* (articulating the standard of review for findings of an interference-in-fact under *35 U.S.C. § 291*). Here, because we agree with the district court's subsidiary finding of obviousness, which is sufficient to support its finding of an interference-in-fact, it is not necessary for us to review the court's finding of anticipation.

[HN3] Obviousness under *35 U.S.C. § 103* is a legal conclusion that is reviewed de novo; however, it is based in turn on underlying factual determinations which are reviewed for clear error. Id. Under the clear error standard, a reversal is permitted "only when this court is left with a 'definite and firm conviction' that the district court was in error." *Ruiz v. A.B. Chance Co., 357 F.3d 1270, 1275 (Fed. Cir. 2004)* (quoting *Amhil Enters. Ltd. v. Wawa, Inc., 81 F.3d 1554, 1562 (Fed. Cir. 1996)).*

2. Obviousness

[HN4] The ultimate determination of whether an invention would have been obvious under *35 U.S.C. § 103* [**14] *(a)* is a legal conclusion based on the factual Graham findings, e.g., "(1) the scope and content of the prior art; (2) the level of ordinary skill in the prior art; and (3) the differences between the claimed invention and the prior art." *Velander v. Garner, 348 F.3d 1359, 1363 (Fed. Cir. 2003)* (citing *Graham v. John Deere Co., 383 U.S. 1, 17, 86 S. Ct. 684, 15 L. Ed. 2d 545 (1966)).*

This court has held that [HN5] if all the elements of an invention are found in a combination of prior art references:

> a proper analysis under *§ 103* requires, inter alia, consideration of two factors: (1) whether the prior art would have suggested to those of ordinary skill in the art that they should make the claimed composition or device, or carry out the claimed process; and (2) whether the prior art would also have revealed that in so making or carrying out, those of ordinary skill would have a reasonable expectation of success.

Id.

The first requirement, the motivation to combine references, serves to prevent hindsight bias. See *McGinley v. Franklin Sports, Inc., 262 F.3d 1339, 1351 (Fed. Cir. 2001)* ("To prevent hindsight invalidation of patent [**15] claims, the law requires some 'teaching, suggestion or reason' to combine cited references.") (quoting *Gambro Lundia AB v. Baxter Healthcare Corp., 110 F.3d 1573 (Fed. Cir. 1997)).*[HN6] In making obviousness determinations, the test is "whether the subject matter of the claimed inventions would have been obvious to one skilled in the art at the time the inventions were made, *not* what would be obvious to a judge after reading the patents in suit and hearing the testimony." *Panduit Corp. v. Dennison Mfg. Co., 774 F.2d 1082, 1092 (Fed. Cir. 1985).* Whether such a motivation [*1165] has been demonstrated is a question of fact. See *Winner Int'l Royalty Corp. v. Wang, 202 F.3d 1340, 1348 (Fed. Cir. 2000).* Evidence of a motivation to combine prior art references "may flow from the prior art references themselves, the knowledge of one of ordinary skill in the art, or, in some cases, from the nature of the problem to be solved." *Brown & Williamson Tobacco Corp. v. Philip Morris Inc., 229 F.3d 1120, 1125 (Fed. Cir. 2000).*

[HN7] When a piece of prior art "suggests that the line of development flowing from the reference's disclosure is unlikely [**16] to be productive of the result sought by the applicant" the piece of prior art is said to "teach away" from the claimed invention. *In re Gurley, 27 F.3d 551, 553 (Fed. Cir. 1994).* As with other subsidiary obviousness inquiries, "what a reference teaches and whether it teaches toward or away from the claimed invention are questions of fact." *Winner, 202 F.3d at 1349* (internal quotations omitted). However, obviousness must be determined in light of all the facts, and there is no rule that a single reference that teaches away will mandate a finding of nonobviousness. Likewise, a given course of action often has simultaneous advantages and disadvantages, and this does not necessarily obviate motivation to combine. See *id. at 1349 n.8* ("The fact that the motivating benefit comes at the expense of another benefit, however, should not nullify its use as a basis to modify the disclosure of one reference with the teachings of another. Instead, the benefits, both lost and gained, should be weighed against one another."). Where the prior art contains "apparently conflicting" teachings (i.e., where some references teach the combination and others [**17] teach away from it) each reference must be considered "for its power to suggest solutions to an artisan of ordinary skill. . . . considering the degree to which one reference might accurately discredit another." *In re Young, 927 F.2d 588, 591 (Fed. Cir. 1991).*

As stated above, an obviousness determination requires not only the existence of a motivation to combine elements from different prior art references, but also that a skilled artisan would have perceived a reasonable expectation of success in making the invention via that combination. While the definition of "reasonable expectation" is somewhat vague, our case law makes clear that it does not require a certainty of success. See *In re O'Farrell, 853 F.2d 894, 903-04 (Fed. Cir. 1988)* [HN8] ("Obviousness does not require absolute predictability of success. . . . All that is required is a reasonable expectation of success.").

However, [HN9] to have a reasonable expectation of success, one must be motivated to do more than merely to "vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters [**18] were critical or no direction as to which of many possible choices is likely to be successful." *Id. at 903.* Similarly, prior art fails to provide the requisite "reasonable expectation" of success where it teaches merely to pursue a "general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it." Id.

[HN10] The district court's finding of a reasonable expectation of success is a question of fact, which we review for clear error. See *Ruiz, 357 F.3d at 1275* (explaining that the obviousness determination rests on "various factual findings that this court reviews for clear error following a bench trial"); *Brown & Williamson, 229 F.3d at 1129* [*1166] (reviewing the district court's finding of reasonable expectation of success under the clear error standard); see also *Velander v. Garner, 348 F.3d 1359, 1376 (Fed. Cir. 2003)* (reviewing the Board of Patent Appeals and Interferences' finding of a reasonable expectation of success under a "substantial evidence" standard).

3. Analysis

Rolabo argues that the [**19] district court erred in finding that the Medichem invention (which uses a tertiary amine) would have been obvious over the broader Rolabo invention (which does not require it). Specifically, it appears to argue both that the prior art contained no motivation to combine references so as to have encouraged one reasonably skilled in the art to have added a tertiary amine to a McMurry reaction and that an artisan, even if motivated to add a tertiary amine to Rolabo's process, would have had no reasonable expectation of

succeeding in making loratadine via a McMurry reaction in the presence of a tertiary amine.

In support of its arguments, Rolabo cites the trial testimony of an expert witness who explained that a seminal review article in the field showed that a tertiary amine could have "a positive effect, a negative effect, and in some cases, both a positive and negative effect" on the McMurry reaction. Rolabo goes on to cite prior art references that disclose negative effects and essentially argues that the existence of prior art references that teach away from the invention clearly negates the motivation to combine and that the district court's finding of motivation was clearly erroneous. [**20] We disagree.

Granted, it is clear that the prior art disclosed not only potential advantages of using a tertiary amine in a McMurry reaction but also potential disadvantages. On the one hand, some pieces of prior art taught that low concentrations of a tertiary amine could sometimes be used to improve the yield of reactions or to avoid the formation of undesirable rearranged products. On the other hand, other references reported that tertiary amines could sometimes promote the formation of undesirable diol side-products and that when they were used as the reaction solvent (i.e., when tertiary amines are present at their highest possible concentrations), they could stop the reaction completely.

We also note the ambivalence of Medichem co-inventor Dr. Onrubia toward the introduction of a tertiary amine to the reaction mixture. On the one hand, she testified that she had added a tertiary amine "because the literature said that it might be possible to use tertiary amines in the reaction, that it wouldn't interfere, that it wasn't incompatible, and it's habitual in these circumstances to try various options until you get the reaction to work." On the other hand, when asked, "Is this purely [**21] hit or miss or is there some logical cause . . . for believing that tertiary amine would add something?" she responded: "Frankly, as an organic chemist I have no reason to say that there were grounds for expecting anything from the addition of tertiary amine."

As we have explained above, the fact that some teachings in the prior art conflict with others does not render the findings of the district court clearly erroneous per se. Rather, the prior art must be considered as a whole for what it teaches. We understand the prior art, viewed as a whole, to teach that the addition of a tertiary amine sometimes works to improve the yield of McMurry reactions, especially when a tertiary amine is used in relatively low concentrations. In light of this, we cannot say that the district court clearly erred in finding that the prior art would have provided the skilled artisan with a [*1167] motivation to combine references so as to use pyridine in the McMurry reaction. We wish to emphasize that this is not a case where the prior art's lack of definiteness or certainty about the result of using a tertiary amine in a specific reaction system renders the inventive subject matter "obvious to [**22] try" but not obvious. While we have made clear that "'obvious to try' is not the standard under *§ 103*, . . . the meaning of this maxim is sometimes lost." *In re O'Farrell, 853 F.2d 894, 903 (Fed. Cir. 1988)*. In O'Farrell, we opined that:

> [This] admonition . . . has been directed mainly at two kinds of error[, namely where] . . . what would have been "obvious to try" would have been . . . to vary all parameters or try each of numerous possible choices . . . where the prior art gave . . . no direction as to which of many possible choices is likely to be successful[or] . . . to explore . . . a promising field of experimentation, where the prior art gave only general guidance . . . .

Id. (citations omitted). In the instant case there are not numerous parameters to vary. Rather, the principal parameter is the concentration of tertiary amine that should be used, and the prior art teaches that if the tertiary amine were to have any positive effect at all, it would be when it was present at low concentrations. Likewise, this is not a case where the prior art gives merely general guidance. In contrast, the guidance is quite clear-namely, that [**23] McMurry reactions of this kind can sometimes be optimized by adding low levels of a tertiary amine.

For the aforementioned reasons, we find no clear error in the district court's determination that skilled artisans in possession of the Rolabo patent and the prior art would have not only been motivated to add a tertiary amine but that they would have possessed a reasonable expectation that they would succeed in optimizing the reaction. Reviewing de novo the trial court's application of these factual findings to reach the legal conclusion of obviousness, we likewise find no error. Accordingly, we agree with the district court's determination that the addition of a tertiary amine to a McMurry reaction would have been obvious in view of the Rolabo patent and the prior art. Because this obviousness finding satisfies the second prong of the two-way test for an interference-in-fact, we affirm the district court's determination that an interference-in-fact existed.

As a final matter, we note that we find no merit in Rolabo's contention that we should exclude from the subject matter of the interference that portion of its invention that is directed to running reactions where titanium [**24] is present in specific concentration ranges

(claims 10 and 11 of the *'827 patent*). Claim 10 requires a relative titanium concentration of 1.5:1 to 4:1, and claim 11 requires a ratio of 2:1 to 3:1. The district court relied on the testimony of Medichem's expert witness, Dr. Finney, in holding that all of the various claims of the *'827 patent* were "essentially identical to one another and substantially the same as claim 2 of Medichem's patent." See *Medichem III, 2004 U.S. Dist. LEXIS 23697, 2004 WL 2674632 at *4*. Rolabo argues that Finney's expert testimony was "conclusory" and therefore insufficient to establish an interference. However, it is clear from the record that Finney's testimony was far from conclusory. In fact, Finney provided a solid factual basis for his opinion, stating that

> "claim 10 says that you should have between, a ratio of one and a half to 4 to 1 titanium to dibenzosuberone. Claim 11 states the range should be 2 to 1 to 3 to 1. These are both perfectly normal ranges. And in fact, the patent examples in the '827 [Rolabo's] patent specify I think about a 2.2 to 1 ratio. . . ."

[*1168] Indeed, other evidence of record also supports the conclusion that these are normal [**25] ranges. The Banerji reference discloses ratios of 2:1 and 1:1, Ishida discloses ratios of 1.5:1, 2.5:1 and 5:1, and Lenoir discloses a ratio of about 1:1.

In short, it is clear that Rolabo's claims 10 and 11 are directed to titanium ratios that are entirely within the range of the prior art, and this fact is dispositive. This court has held that "selecting a narrow range from within a somewhat broader range disclosed in a prior art reference is no less obvious than identifying a range that simply overlaps a disclosed range." *In re Peterson, 315 F.3d 1325, 1330 (Fed. Cir. 2003)*. Moreover, when "the claimed ranges are completely encompassed by the prior art, the conclusion is even more compelling than in cases of mere overlap." *Id.* We have explained that the "normal desire of scientists or artisans to improve upon what is already generally known provides the motivation to determine where in a disclosed set of percentage ranges is the optimum combination of percentages." Id. Therefore, because Rolabo's claims 10 and 11 are directed to ratios that are entirely within the prior art, the district court properly held those claims to be part of the interfering subject [**26] matter pursuant to the two-way test.

B. Identification of Interfering Subject Matter

Having affirmed the district court's determination that an interference-in-fact exists, and that it properly includes those claims directed to specific titanium ratios, this court turns to address Rolabo's procedural argument that the district court erred when it failed to comply with the Board's practice of articulating a precise count of the interference prior to making priority determinations.

This court has not yet addressed "whether district courts handling interfering patent suits under *§ 291* must define this interfering subject matter in a way similar to a count." *Slip Track Sys., Inc. v. Metal-Lite, Inc., 304 F.3d 1256, 1264 (Fed. Cir. 2002)*. Nevertheless, we have made clear that [HN11] at least "a single description of the interfering subject matter is necessary for a determination of priority." Id.

That said, *SlipTrack* does not require a court to refer explicitly to the interfering subject matter as a "count," and we believe that in this case the district court was clear about the identity of the interfering subject matter, stating in its opinion "all the various claims [**27] of the *'827 patent* are essentially identical to one another and substantially the same as claim 2 of Medichem's patent." *Medichem III, 2004 U.S. Dist. LEXIS 23697, 2004 WL 2674632 at *4*. Moreover, to the extent that the district court may not have been clear about whether the tertiary amine limitation was part of the interfering subject matter, we can resolve this issue on appeal. See *Slip Track, 304 F.3d at 1264-65* (holding that where "the parties . . . dispute only whether one limitation is part of the interfering subject matter, and determination of this issue is dependent upon issues of law alone, we will resolve this issue on appeal.") Accordingly, we hold that the interfering subject matter in this case does not include the limitation of the tertiary amine, and corresponds to claim 17 of Rolabo's *'827 patent*. See *id. 1265* ("Since the claims of the '760 patent do not include a wallboard . . . the wallboard cannot be an element of the interfering subject matter in this case, even though it is a limitation in the claims of the '203 patent."). [7]

7 We note that in parallel interference proceedings, pursuant to *35 U.S.C. § 135*, the Board reached a similar definition of the count. See *Stampa v. Jackson, 2002 Pat. App. LEXIS 191, 65 U.S.P.Q.2d 1942, 1948 (B.P.A.I. 2002)* (defining the count as Jackson's (Rolabo's) claim 17).

[**28] [*1169] C. Priority of Invention

Finally, we review the district court's award of priority of invention to Medichem. Because the Medichem *'100 patent* issued from an application that had a later effective filing date than did Rolabo's *'827 patent* application, see supra note 4, Medichem bears the burden of establishing priority by a preponderance of the evidence. See *Eli Lilly & Co. v. Aradigm Corp., 376 F.3d 1352, 1365 (Fed. Cir. 2004)* [HN12] ("Under *35 U.S.C. § 291*, a party that does not have the earliest effective filing date

needs only to demonstrate by a preponderance of the evidence that it was the first to invent if the two patents or applications at issue were co-pending before the PTO . . . ."). Medichem bears no heightened burden, because neither patent enjoys a statutory presumption of validity. See id. ("The presumption of validity is nonexistent and the preponderance of the evidence burden is appropriate even if both of the patents have issued by the time a *section 291* interference proceeding is initiated in a district court.").

We have held that [HN13] "priority of invention goes to the first party to reduce an invention to practice unless the [**29] other party can show that it was the first to conceive of the invention and that it exercised reasonable diligence in later reducing that invention to practice." *Cooper v. Goldfarb, 154 F.3d 1321, 1327 (Fed. Cir. 1998).* Here, because [HN14] neither party relied on a date of conception, priority is properly awarded to the party that was the first to reduce its invention to practice, either actually or constructively. Rolabo relies on its date of constructive reduction to practice, namely its February 26, 1997 effective filing date. Medichem, on the other hand, alleges that it achieved an actual reduction to practice in the spring of 1996, a date which if proven would antecede Rolabo's filing date, and thereby entitle it to priority. See supra note 4 (effective filing dates).

[HN15] In order to establish an actual reduction to practice, Medichem must establish three things: "(1) construction of an embodiment or performance of a process that met all the limitations of the interference count; (2) . . . determination that the invention would work for its intended purpose," *Cooper, 154 F.3d at 1327*; and (3) the existence of sufficient evidence to corroborate [**30] inventor testimony regarding these events, see *id. at 1330* ("In order to establish an actual reduction to practice, an inventor's testimony must be corroborated by independent evidence."). The key issue on appeal is the last one, namely whether Medichem provided adequate corroboration of the inventors' testimony regarding the alleged actual reduction to practice.

For purposes of conceptual clarity, as well as clarity of language, it should be noted that [HN16] no similar condition of "corroboration" is imposed on an inventor's notebook, or indeed on any documentary or physical evidence, as a condition for its serving as evidence of reduction to practice. See, e.g., *Mahurkar v. C.R. Bard, Inc., 79 F.3d 1572, 1577-78 (Fed. Cir. 1996)* (explaining that "this court does not require corroboration where a party seeks to prove conception through the use of physical exhibits because the trier of fact can conclude for itself what documents show, aided by testimony as to what the exhibit would mean to one skilled in the art"); *Price v. Symsek, 988 F.2d 1187, 1195 (Fed. Cir. 1993)* ("Only the inventor's testimony requires corroboration [*1170] before it [**31] can be considered."). Of course, the credibility (and therefore the corroborative value) of an inventor's notebook may vary. Nevertheless, a notebook, unlike the oral testimony of an inventor, may be weighed, for whatever it is worth, in the final determination of reduction to practice. However, in a case involving reduction to practice, an unwitnessed notebook is insufficient on its own to support a claim of reduction to practice. See *Reese v. Hurst, 661 F.2d 1222, 1232 (CCPA 1981)* ("The inventors' notebooks are accorded no more weight than the inventors' testimony in this instance, since they were not witnessed or signed and were unseen by any witness until after this interference was declared."); *Hahn v. Wong, 892 F.2d 1028, 1033 (Fed. Cir. 1989)* (stating that "affiants' statements that by a certain date they had 'read and understood' specified pages of Stephen Hahn's laboratory notebooks did not corroborate a reduction to practice . . . because they established only that those pages existed on a certain date . . . [and] did not independently corroborate the statements made on those pages); *Singh v. Brake, 222 F.3d 1362, 1370 (Fed. Cir. 2000)* [**32] (stating that Hahn v. Wong did not nullify the value of laboratory notebooks in corroborating conception because "the standard of proof required to corroborate a reduction to practice [is] more stringent . . . than that required to corroborate a conception."). [8] Once properly admitted into evidence, documentary and physical evidence is assigned probative value and collectively weighed to determine whether reduction to practice has been achieved. This is what is meant by the maxim that documentary and physical evidence do not require "corroboration."

8 Cf. *Stern v. Trs. of Columbia Univ., 434 F.3d 1375, 2006 U.S. App. LEXIS 1015, No. 05-1291, slip op. at 5 (Fed. Cir. Jan. 17, 2006)* ("Regardless of the contents of the notebooks, unwitnessed laboratory notebooks on their own are insufficient to support his claim [of conception, and therefore] of co-inventorship.").

1. Corroboration

Credibility concerns undergird the corroboration requirement, the purpose of which is to prevent fraud. See *Chen v. Bouchard, 347 F.3d 1299, 1309 (Fed. Cir. 2003)* ("The purpose of corroboration . . . is to prevent fraud, by providing independent confirmation of the inventor's testimony.") (internal [**33] quotations omitted). As such, the corroboration requirement provides an additional safeguard against courts being deceived by inventors who may be tempted to mischaracterize the events of the past through their testimony. See *Mahurkar, 79 F.3d at 1577* ("While perhaps prophylactic in application given the unique abilities of trial court judges and juries

to assess credibility, the rule provides a bright line for both district courts and the PTO to follow in addressing the difficult issues related to invention dates.").

[HN17] Sufficiency of corroboration is determined by using a "rule of reason" analysis, under which all pertinent evidence is examined when determining the credibility of an inventor's testimony. See *Price v. Symsek, 988 F.2d 1187, 1195 (Fed. Cir. 1993)* ("'A rule of reason' analysis is applied to determine whether the inventor's prior conception testimony has been corroborated."); *Berges v. Gottstein, 618 F.2d 771, 776 (CCPA 1980)* ("In the final analysis, each corroboration case must be decided on its own facts with a view to deciding whether the evidence as a whole is persuasive.").

[HN18] The requirement of independent knowledge [**34] remains key to the corroboration inquiry. See *Reese v. Hurst, 661 F.2d 1222, 1225 (CCPA 1981)* ("Adoption of the 'rule of reason' has not altered the [*1171] requirement that evidence of corroboration must not depend solely on the inventor himself."). "Independent corroboration may consist of testimony of a witness, other than the inventor, to the actual reduction to practice or it may consist of evidence of surrounding facts and circumstances independent of information received from the inventor." *Id.* One consequence of the independence requirement is that testimony of one co-inventor cannot be used to help corroborate the testimony of another. See, e.g., *Lacks Indus. v. McKechnie Vehicle Components USA, Inc., 322 F.3d 1335, 1350 (Fed. Cir. 2003)* (opining that the Special Master rightly refused to accept cross-corroboration of oral testimony as being adequate).

Despite the importance of the independence requirement, however, [HN19] "the law does not impose an impossible standard of 'independence' on corroborative evidence by requiring that every point of a reduction to practice be corroborated by evidence having a source totally independent of the inventor. [**35] . . ." *Cooper v. Goldfarb, 154 F.3d at 1330* (internal quotations omitted). Similarly, "it is not necessary to produce an actual over-the-shoulder observer. Rather, sufficient circumstantial evidence of an independent nature can satisfy the corroboration requirement." Id.

[HN20] When an inventor claims a process for making a chemical compound rather than the compound itself, it is the successful reduction to practice of the process that must be corroborated, and not merely the successful production of the compound per se. Thus, spectral evidence that might be sufficient per se to corroborate a claim directed to the product will generally not be sufficient to corroborate a claim directed to the process, in the absence of some evidence to corroborate that the product was produced via that process.

2. Standard of Review

[HN21] Whether or not corroboration exists is a question of fact, the district court's determination of which we review for clear error. This is true because "issues of conception and reduction to practice are questions of law predicated on subsidiary factual findings," *Eaton v. Evans, 204 F.3d 1094, 1097 (Fed. Cir. 2000)*, and corroboration [**36] is properly viewed as a subsidiary factual finding. See *Singh v. Brake, 222 F.3d at 1368* (implying that corroboration is a question of fact by holding that "substantial evidence supports the Board's finding that this notebook entry alone was insufficient to corroborate Singh's testimony . . . .")(emphasis added).

Before reviewing the determination of the court below, we note that it is true that [HN22] corroboration is fundamentally about "credibility," see supra Discussion, Part C.1, and that in reviewing factual findings under the clear error standard, this court "gives great deference to the district court's decisions regarding credibility of witnesses." See *Ecolochem, Inc. v. S. Cal. Edison Co., 227 F.3d 1361, 1378-79 (Fed. Cir. 2000)* (internal quotations omitted). Indeed, such deference is appropriately accorded to assessments of witness credibility because "only the trial judge can be aware of the variations in demeanor and tone of voice that bear so heavily on the listener's understanding of and belief in what is said." *Anderson v. Bessemer City, 470 U.S. 564, 575, 105 S. Ct. 1504, 84 L. Ed. 2d 518 (1985).*

Nonetheless, such deference is often [**37] of little consequence in a corroboration inquiry because the raison d'etre of the corroboration requirement is our refusal to base priority determinations on a court's uncorroborated assessments of a testifying inventor's credibility.[HN23] Even the most credible inventor testimony is a fortiori required to be corroborated by independent [*1172] evidence, which may consist of documentary evidence as well as the testimony of non-inventors. To the extent that a district court's finding of corroboration rests on its assessment of the credibility of non-inventor testimony, we apply the deferential standard of review stated in *Ecolochem*. To the extent that it rests, as it does here, on the district court's assessment of documentary, as opposed to testimonial evidence, we still apply clear error review; however, clear error is less difficult to establish.

3. Analysis

The parties in this case dispute whether or not there was adequate corroboration of the inventors' testimony that Medichem had actually reduced to practice the process of the claimed invention before Rolabo's effective filing date. Medichem put forward two principal types of corroborating evidence: documentary evidence generated [**38] by inventors and that generated by non-inventors. [9]

9 This patent bore a number of co-inventors, many of whom testified at trial. As we have noted above, the testimony of one inventor cannot be corroborated by the testimony of co-inventors.

In the first category, it produced a documented request for the analysis of a sample, purported to have been produced via the claimed synthetic route, which was sent by one co-inventor to another. Also in this category were the NMR spectral data obtained by the co-inventor pursuant to that request. These spectra were consistent with loratadine, and the accuracy of that chemical identification is not being challenged. Finally, this category includes the original laboratory notebook of co-inventor Dr. Rodriguez. In the second category, documentary evidence by non-inventors, there is the original laboratory notebook of former Medichem employee, and non-inventor, Lola Casas.

This court now turns to consider the corroborative value of the three principal pieces of potentially corroborative evidence: the NMR spectra, the notebooks of Medichem's inventors, and the notebook of non-inventor Casas. We note at the outset that the [**39] problem with the dated NMR data is that at most they corroborate that the inventors were in possession of the chemical loratadine as of that date; they do not, in themselves, adequately corroborate the claimed process, as they do not establish whether the sample that was analyzed was actually produced by that process. If this case dealt with a claim to a composition of matter, rather than to a process, the NMR evidence might very well take on a different relevance in this regard. As far as the corroborative value of the inventors' notebooks is concerned, they were not witnessed, and they do not provide an "independent" source of authority on the issue of reduction to practice. Hence, they have minimum corroborative value.

It is clear to this court, therefore, that Medichem's claim of corroboration stands or falls with the modicum of additional corroborative value that can properly be assigned to non-inventor Casas' notebook. [10] However, Casas did not testify [*1173] regarding the notebook or the genuineness of its contents. In addition, although Casas' notebook was dated, it was neither signed nor witnessed, and inventor Rodriguez testified that she and Casas had made entries in each [**40] others' notebooks. Rodriguez characterized these occasions as not out of the ordinary. As a result, the district court was clearly reliant on the inventor to help to identify the author of specific entries made in Casas' notebook, because in a reduction to practice inquiry, only those passages of the unsigned, unwitnessed notebooks authored by non-inventor Casas could possess significant corroborative value. In addition, without testimony from Casas, the court lacked any non-inventor testimony regarding the genuineness of the notebook's contents.

10 When an inventor attempts to offer into evidence the notebook of a non-inventor as evidence of corroboration, evidentiary issues might be implicated. For example, the notebook is likely to be hearsay, and if so, there may be an issue as to whether or not it falls within an exception to the hearsay rule, such as the business record exception. Indeed, in Chen v. Bouchard, this court affirmed the decision of the Board of Patent Appeals and Interferences to exclude as inadmissible hearsay a non-inventor's notebooks, which had been offered to corroborate reduction to practice where, as in the instant case, the non-inventor did not testify. *347 F.3d 1299, 1308 (Fed. Cir. 2003).*

[**41] We also note that Medichem admitted fraudulently backdating certain documents relating to a purported 1995 reduction to practice. Even though the backdating of the 1995 documents was unrelated to the critical pages in Casas' notebook, which purport to establish a reduction to practice in 1996, the district court found that the credibility of the Medichem inventors was accordingly diminished.

[HN24] Where a laboratory notebook authored by a non-inventor is offered into evidence pursuant to authentication by an inventor, where the author of the notebook has not testified at trial or otherwise attested to its authenticity, and where the notebook has not been signed or witnessed and has not been maintained in reasonable accordance with good laboratory practices sufficient to reasonably ensure its genuineness under the circumstances, then the corroborative value of the notebook is minimal. Given the facts of this case, Casas' notebook should therefore not be accorded much corroborative value. In view of the minimal corroborative value of the inventors' notebooks and the limited value of the NMR spectrum, we conclude that the evidence, evaluated as a whole under the rule of reason, is [**42] insufficient as a matter of law to corroborate Medichem's reduction to practice.

The district court did not specifically address corroboration in its obviousness inquiry, a fact that might, in some circumstances, hamper our ability to conduct clear error review. Here, however, the facts of the case admit of only one conclusion as a matter of law, and we therefore decide the case without remanding to the district court for an explanation of why it implicitly found corroboration to be present. We hold that corroboration is absent and that the district court therefore erred in reaching its legal conclusion that Medichem had reduced its invention to practice in the spring of 1996. Accordingly,

we reverse the district court's award of priority to Medichem.

AFFIRMED-IN-PART, REVERSED-IN-PART

No costs.

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U.S. Appln. No. 10/770,639
Reference No. 12

LEXSEE 383 U.S. 1

GRAHAM ET AL. v. JOHN DEERE CO. OF KANSAS CITY ET AL.

No. 11

SUPREME COURT OF THE UNITED STATES

383 U.S. 1; 86 S. Ct. 684; 15 L. Ed. 2d 545; 1966 U.S. LEXIS 2908; 148 U.S.P.Q. (BNA) 459

October 14, 1965, Argued
February 21, 1966, Decided *

* Together with No. 37, Calmar, Inc. v. Cook Chemical Co., and No. 43, Colgate-Palmolive Co. v. Cook Chemical Co., also on certiorari to the same court.

PRIOR HISTORY: CERTIORARI TO THE UNITED STATES COURT OF APPEALS FOR THE EIGHTH CIRCUIT.

DISPOSITION: *333 F.2d 529*, affirmed; *336 F.2d 110*, reversed and remanded.

CASE SUMMARY:

PROCEDURAL POSTURE: Two decisions of the United States Court of Appeals for the Eighth Circuit were consolidated on appeal to consider the effect of the Patent Act of 1952, *35 U.S.C.S. § 103*, on the validity of patents. One decision granted judgment for respondents in petitioners' patent infringement suit, and the other affirmed judgment for respondent, who brought cross actions for infringement in petitioners' consolidated declaratory judgment actions.

OVERVIEW: Two appeals were consolidated to review the validity of patents in light of the Patent Act of 1952, *35 U.S.C.S. § 103*, which provided that in determining the patentability of a device it was necessary to consider not only its novelty and utility, but also its obviousness to one of ordinary skill in the art. The court affirmed a circuit court judgment in favor of respondents in petitioners' plow clamp patent infringement suit, and reversed a decision affirming judgment for respondent, who brought cross actions for infringement of its sprayer patent in petitioners' consolidated declaratory judgment suits. On appeal, the court held that *§ 103* placed an emphasis on the factor of obviousness but did not lower the level of patentable invention. The court then examined the patents in question in light of the prior art and determined that the plow clamp patent in the first case was invalid because there were no operative mechanical distinctions, much less nonobvious differences in petitioners' device, and the sprayer patent in the second two cases was also invalid because its invention rested on small, nontechnical differences in a device that was otherwise old in the art.

OUTCOME: The court affirmed judgment in favor of respondent plow manufacturers and reversed judgments in favor of respondent and its patented sprayer, applying its finding that the Patent Act of 1952 did not change the general level of patentable invention, and holding that the inventions in question were invalid because their obviousness would be evident to one of ordinary skill in the pertinent art.

LexisNexis(R) Headnotes

Patent Law > Nonobviousness > Elements & Tests > Claimed Invention as a Whole
Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN1] See *35 U.S.C.S. § 103.*

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN2] Under the Patent Act of 1952, *35 U.S.C.S. § 103*, patentability is to depend, in addition to novelty and utility, upon the non-obvious nature of the subject matter

sought to be patented to a person having ordinary skill in the pertinent art.

Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > Nonobviousness > Elements & Tests > Prior Art
Patent Law > Nonobviousness > Elements & Tests > Secondary Considerations
[HN3] While the ultimate question of patent validity is one of law, the condition set forth in the Patent Act of 1952, *35 U.S.C.S. § 103*, which is but one of three conditions, each of which must be satisfied, lends itself to several basic factual inquiries: (1) the scope and content of the prior art are to be determined; (2) differences between the prior art and the claims at issue are to be ascertained, and (3) the level of ordinary skill in the pertinent art resolved. Against this background, the obviousness or nonobviousness of the subject matter is determined. Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented. As indicia of obviousness or nonobviousness, these inquiries may have relevancy.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN4] Although the inquiry which the U.S. Patent Office and the courts must make as to patentability must be beamed with greater intensity on the requirements of the Patent Act of 1952, *35 U.S.C.S. § 103*, there is no change in the general strictness with which the overall test is to be applied.

Patent Law > Infringement Actions > Claim Interpretation > General Overview
Patent Law > Infringement Actions > Prosecution History Estoppel > General Overview
[HN5] An invention is construed not only in the light of the claims, but also with reference to the file wrapper or prosecution history in the U.S. Patent Office. Claims as allowed must be read and interpreted with reference to rejected ones and to the state of the prior art; and claims that have been narrowed in order to obtain the issuance of a patent by distinguishing the prior art cannot be sustained to cover that which was previously by limitation eliminated from the patent.

SUMMARY:

In No. 11, the patentee and a licensee under a patent on a clamp for vibrating shank plows brought a patent infringement suit in the United States District Court for the Western District of Missouri. The District Court found the patent valid and infringed (*216 F Supp 272*), but the United States Court of Appeals for the Eighth Circuit reversed. (*333 F2d 529.*) In Nos. 37 and 43, consolidated actions brought in the United States District Court for the Western District of Missouri for judgments declaring invalid a patent on a plastic finger sprayer with a hold-down overcap, the patent was sustained by the *District Court (220 F Supp 414)*, and the United States Court of Appeals for the Eighth Circuit affirmed. (*336 F2d 110.*)

On certiorari, the Supreme Court of the United States affirmed in No. 11 and reversed in Nos. 37 and 43. In an opinion by Clark, J., expressing the unanimous view of the Court, it was held that (1) 103 of the Patent Act of 1952, providing that a patent may not be obtained if the subject matter would have been obvious to a person with ordinary skill in the art, codified existing judicial precedents without affecting the required general level of innovation, and (2) both patents were invalid under 103, since the differences between them and the pertinent prior art would have been obvious to a person reasonably skilled in that art.

Stewart, J., did not participate in Nos. 37 and 43.

Fortas, J., did not participate in any of the cases.

LAWYERS' EDITION HEADNOTES:

[***LEdHN1]

PATENTS §19

patentability -- mechanical skill --

Headnote:[1A][1B]

The Patent Act of 1952 (66 Stat 792, ch 950) is intended to codify judicial precedents embodying the principle that a patentable invention must evidence more ingenuity and skill than that possessed by an ordinary mechanic acquainted with the business.

[***LEdHN2]

PATENTS §19.1

patentability -- obviousness --

Headnote:[2A][2B]

Although 103 of the Patent Act of 1952 (*35 USC 103*) places emphasis on an inquiry into obviousness by providing that a patent may not be obtained if the subject matter "would have been obvious at the time the invention was made to a person having ordinary skill in the art," the general level of innovation necessary to sustain

the patentability remains the same as before the statute was enacted.

[***LEdHN3]

PATENTS §2

congressional power -- constitutional limitations --

Headnote:[3]

Article 1 8 of the Constitution, authorizing Congress to promote the progress of the useful arts by securing for limited times to inventors the exclusive right to their discoveries, is both a grant of power and a limitation; the limitation to the promotion of advances in the "useful arts" precludes Congress from enlarging the patent monopoly without regard to the innovation, advancement, or social benefit gained thereby and from authorizing the issuance of patents whose effects are to remove existent knowledge from the public domain or to restrict free access to materials already available.

[***LEdHN4]

PATENTS §140

validity --

Headnote:[4]

The question of patent validity requires reference to a standard written into the Constitution.

[***LEdHN5]

PATENTS §2

congressional power --

Headnote:[5]

Within the scope and limits of Article 1 8 authorization to Congress to promote the useful arts by securing for limited times to inventors the exclusive right to their discoveries, Congress may set out conditions and tests for patentability.

[***LEdHN6]

UNITED STATES §16

congressional powers --

Headnote:[6]

As a corollary to the grant of any power under Article 1 of the Constitution, Congress may implement the stated constitutional purpose by selecting the policy which in its judgment best effectuates the constitutional aim.

[***LEdHN7]

COURTS §118.5

PATENTS §3

effectuating congressional scheme --

Headnote:[7]

It is the duty of the Commissioner of Patents and of the courts in the administration of the patent system to give effect to the constitutional standard by appropriate application, in each case, of the statutory scheme of Congress.

[***LEdHN8]

PATENTS §18

PATENTS §19.1

PATENTS §55

conditions of patentability --

Headnote:[8]

Under the Patent Act of 1952 (66 Stat 792, ch 950), patentability is dependent upon three explicit conditions: novelty and utility, as articulated and defined in *35 USC 101* and *102*, and nonobviousness, as set out in *35 USC 103*.

[***LEdHN9]

PATENTS §16

patentability -- flash of genius --

Headnote:[9]

The provision of *35 USC 103* that "patentability shall not be negatived by the manner in which the invention was made" is intended to abolish the test of patentability expressed in the controversial phrase "flash of creative genius."

[***LEdHN10]

PATENTS §19.1

test of obviousness --

Headnote:[10]

The condition of patentability announced in *35 USC 103*, providing that a patent may not be obtained if the subject matter "would have been obvious at the time the invention was made to a person having ordinary skill in the art," emphasizes nonobviousness in the light of inquiry, not quality, and as such comports with the constitutional strictures.

[***LEdHN11]

PATENTS §19.1

TRIAL §154

patent validity -- obviousness --

Headnote:[11]

While the ultimate question of patent validity is one of law, the condition of *35 USC 103* that a patent may not be obtained if the subject matter "would have been obvious at the time the invention was made to a person having ordinary skill in the art" lends itself to several basic factual inquiries, since the obviousness or nonobviousness of the subject matter is determined against the background of the scope and content of the prior art, the differences between the prior art and the claims at issue, and the level of ordinary skill in the pertinent art; moreover, relevant indicia of obviousness or nonobviousness may be found in such secondary considerations as commercial success, long felt but unsolved needs, the failure of others, etc.

[***LEdHN12]

PATENTS §19.1

nonobviousness test --

Headnote:[12]

The nonobviousness test, created by the provision in *35 USC 103* that a patent may not be obtained if the subject matter "would have been obvious at the time the invention was made to a person having ordinary skill in the art," is not likely to be applied with uniformity of thought in every factual context, but should be amenable to a case-by-case development.

[***LEdHN13]

PATENTS §3

unpatentable material --

Headnote:[13]

The primary responsibility for sifting out unpatentable material lies in the Patent Office.

[***LEdHN14]

PATENTS §19.1

patent on shank plow clamp -- obviousness --

Headnote:[14]

A patent on a clamp for vibrating shank plows, which differed from the prior art only in that the shank was placed below rather than above the hinge plate, and the shank was bolted to the hinge plate and passed through a stirrup, with the result that the shank did not wear on the upper plate or the hinge plate, and the shank was permitted to flex more freely, is invalid under *35 USC 103*, which provides that a patent may not be obtained if the subject matter "would have been obvious at the time the invention was made to a person having ordinary skill in the art."

[***LEdHN15]

PATENTS §82

omission from claim --

Headnote:[15A][15B]

Where a feature not specifically claimed in a patent is disclosed in the drawings and specifications, it becomes public property.

[***LEdHN16]

PATENTS §123

claims -- construction --

Headnote:[16]

An invention is construed not only in the light of the claims but also with reference to the file wrapper or prosecution history in the Patent Office.

[***LEdHN17]

PATENTS §128

narrowed claims -- limitation --

Headnote:[17]

Patent claims as allowed must be read and interpreted with reference to rejected ones and to the state of the prior art; and claims that have been narrowed in order to obtain the issuance of a patent by distinguishing the prior art cannot be sustained to cover that which was previously by limitation eliminated from the patent.

[***LEdHN18]

PATENTS §57

closely related art --

Headnote:[18]

Closure devices in such a closely related art as pouring spouts for liquid containers are at the very least pertinent references in determining the patentability of a plastic finger sprayer with a hold-down overcap used as a built-in dispenser for liquid containers, where the claims are limited to an overcap not contacting the container cap and a rib seal for the overcap.

[***LEdHN19]

PATENTS §18

PATENTS §21

patentability -- failure of others -- utility --

Headnote:[19]

Legal inferences from the failure of others to solve a problem, the long- felt need in the industry for the device, and its wide commercial success may lend a helping hand to the judiciary in determining patentability and serve to guard against slipping into hindsight and to resist the temptation to read into the prior art the teachings of the invention in issue.

[***LEdHN20]

PATENTS §21

patentability -- early unsuccessful experiments --

Headnote:[20]

In determining patentability, unsuccessful attempts to reach a solution become wholly irrelevant with the appearance of another patent, before the issuance of the patent in litigation, showing the solution claimed by the patent in litigation.

[***LEdHN21]

PATENTS §61

patentability -- lack of patent search --

Headnote:[21]

In determining patentability, it is irrelevant that no one apparently chose to avail himself of knowledge stored in the Patent Office and readily available by the simple expedient of conducting a patent search.

[***LEdHN22]

PATENTS §19.1

patentability -- obviousness --

Headnote:[22]

The claims of a patent on a plastic finger sprayer with a hold-down overcap fall as not meeting the nonobviousness test of *35 USC 103*, since the differences between them and the pertinent prior art would have been obvious to a person reasonably skilled in that art, where the claims are limited to an overcap not contacting the container cap and a rib seal for the overcap, and both the space and the rib seal were disclosed in a prior patent.

SYLLABUS

In No. 11 petitioners sued for infringement of a patent, consisting of a combination of old mechanical elements, for a device designed to absorb shock from plow shanks in rocky soil to prevent damage to the plow. In 1955 the Fifth Circuit held the patent valid, ruling that a combination is patentable when it produces an "old result in a cheaper and otherwise more advantageous way." Here the Eighth Circuit held that since there was no new result in the combination the patent was invalid. Petitioners in Nos. 37 and 43 filed actions for declaratory judgments declaring invalid respondent's patent relating to a plastic finger sprayer with a "hold-down" cap used as a built-in dispenser for containers with liquids, principally insecticides. By cross-action respondent claimed infringement. The District Court and the Court of Appeals sustained the patent. *Held*: The patents do not meet the test of the "nonobvious" nature of the "subject matter sought to be patented" to a person having ordinary skill in the pertinent art, set forth in *§ 103* of the Patent Act of 1952, and are therefore invalid. Pp. 3-37.

(a) In carrying out the constitutional command of Art. I, § 8, that a patent system "promote the Progress of . . . useful Arts," Congress established the two statutory requirements of novelty and utility in the Patent Act of 1793. Pp. 3, 6, 12.

(b) This Court in *Hotchkiss v. Greenwood, 11 How. 248 (1851)*, additionally conditioned the issuance of a patent upon the evidence of more ingenuity and skill than that possessed by an ordinary mechanic acquainted with the business. P. 11.

(c) In *§ 103* of the 1952 Patent Act Congress added the statutory nonobvious subject matter requirement, originally expounded in *Hotchkiss*, which merely codified judicial precedents requiring a comparison of the subject matter sought to be patented and the prior art, tying patentable inventions to advances in the art. Although *§ 103* places emphasis upon inquiries into obviousness, rather than into "invention," the general level of innovation necessary to sustain patentability remains unchanged under the 1952 Act. Pp. 14-17.

(d) This section permits a more practical test of patentability. The determination of "nonobviousness" is made after establishing the scope and content of prior art, the differences between the prior art and the claims at issue, and the level of ordinary skill in the pertinent art. P. 17.

(e) With respect to each patent involved here the differences between the claims in issue and the pertinent prior art would have been obvious to a person reasonably skilled in that art. Pp. 25-26, 37.

COUNSEL: Orville O. Gold argued the cause for petitioners in No. 11. With him on the brief was Claude A.

Fishburn. Dennis G. Lyons argued the cause for petitioners in Nos. 37 and 43. With him on the briefs for petitioner in No. 37 were Victor H. Kramer and Francis G. Cole. On the brief for petitioner in No. 43 were George H. Mortimer and Howard A. Crawford.

S. Tom Morris argued the cause for respondents in No. 11. With him on the brief were W. W. Gibson and Thomas E. Scofield. Gordon D. Schmidt argued the cause for respondent in Nos. 37 and 43. With him on the brief were Carl E. Enggas, Hugh B. Cox and Charles A. Miller.

Briefs of amici curiae in No. 11 were filed by Roger Robb for the American Bar Association; by Stanton T. Lawrence, Jr., for the New York Patent Law Association; by George E. Frost for the Illinois State Bar Association; by J. Vincent Martin, Alfred H. Evans and Russell E. Schlorff for the State Bar of Texas; and by Robert W. Hamilton for the School of Law of the University of Texas.

JUDGES: Warren, Harlan, Brennan, Black, Clark, White, Douglas; Fortas took no part in the consideration or decision of these cases; Stewart took no part in the consideration or decision of Nos. 37 and 43

OPINION BY: CLARK

OPINION

[*3] [***548] [**686] MR. JUSTICE CLARK delivered the opinion of the Court.

After a lapse of 15 years, the Court again focuses its attention on the patentability of inventions under the standard of *Art. I, § 8, cl. 8, of the Constitution* and under the conditions prescribed by the laws of the United States. Since our last expression on patent validity, *A. & P. Tea Co. v. Supermarket Corp., 340 U.S. 147 (1950)*, the Congress has for the first time expressly added a third statutory dimension to the two requirements of novelty and utility that had been the sole statutory test since the Patent Act of 1793. This is the test of obviousness, *i. e.*, whether "the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made." *§ 103* of the Patent Act of 1952, *35 U. S. C. § 103 (1964 ed.)*.

[***LEdHR1A] [1A] [***LEdHR2A] [2A]

The questions, involved in each of the companion cases before us, are what effect the 1952 Act [***549] had upon traditional statutory and judicial tests of patentability and what definitive tests are now required. We have concluded that the 1952 Act was intended to codify judicial precedents embracing the principle long ago [*4] announced by this Court in *Hotchkiss v. Greenwood, 11 How. 248 (1851)*, and that, while the clear language of *§ 103* places emphasis on an inquiry into obviousness, the general [**687] level of innovation necessary to sustain patentability remains the same.

I.

The Cases.

(a). No. 11, *Graham* v. *John Deere Co.*, an infringement suit by petitioners, presents a conflict between two Circuits over the validity of a single patent on a "Clamp for vibrating Shank Plows." The invention, a combination of old mechanical elements, involves a device designed to absorb shock from plow shanks as they plow through rocky soil and thus to prevent damage to the plow. In 1955, the Fifth Circuit had held the patent valid under its rule that when a combination produces an "old result in a cheaper and otherwise more advantageous way," it is patentable. *Jeoffroy Mfg., Inc. v. Graham, 219 F.2d 511*, cert. denied, *350 U.S. 826*. In 1964, the Eighth Circuit held, in the case at bar, that there was no new result in the patented combination and that the patent was, therefore, not valid. *333 F.2d 529*, reversing *216 F.Supp. 272*. We granted certiorari, *379 U.S. 956*. Although we have determined that neither Circuit applied the correct test, we conclude that the patent is invalid under *§ 103* and, therefore, we affirm the judgment of the Eighth Circuit.

(b). No. 37, *Calmar, Inc.* v. *Cook Chemical Co.*, and No. 43, *Colgate-Palmolive Co.* v. *Cook Chemical Co.*, both from the Eighth Circuit, were separate declaratory judgment actions, but were filed contemporaneously. Petitioner in *Calmar* is the manufacturer of a finger-operated sprayer with a "hold-down" cap of the type commonly seen on grocers' shelves inserted in bottles of insecticides and other liquids prior to shipment. Petitioner in *Colgate-Palmolive* is a purchaser of the sprayers [*5] and uses them in the distribution of its products. Each action sought a declaration of invalidity and noninfringement of a patent on similar sprayers issued to Cook Chemical as assignee of Baxter I. Scoggin, Jr., the inventor. By cross-action, Cook Chemical claimed infringement. The actions were consolidated for trial and the patent was sustained by the District Court. *220 F.Supp. 414*. The Court of Appeals affirmed, *336 F.2d 110*, and we granted certiorari, *380 U.S. 949*. We reverse.

Manifestly, the validity of each of these patents turns on the facts. The basic problems, however, are the

same in each case and require initially a discussion of the constitutional and statutory provisions covering the patentability of the inventions.

II.

[***LEdHR3] [3] [***LEdHR4] [4]At the outset it must be remembered that the federal patent power stems from a specific constitutional provision which authorizes the Congress "To promote the Progress of . . . useful Arts, by securing for limited Times to . . . Inventors the exclusive Right to their . . . Discoveries." Art. I, § 8, cl. 8. [***550] [1] The clause is both a grant of power and a limitation. This qualified authority, unlike the power often exercised in the sixteenth and seventeenth centuries by the English Crown, is limited to the promotion of advances in the "useful arts." It was written against the backdrop of the practices -- eventually curtailed by the Statute of Monopolies -- of the Crown in granting monopolies to court favorites in goods or businesses which had long before been enjoyed by the public. See Meinhardt, Inventions, Patents and Monopoly, pp. [**688] 30-35 (London, 1946). The Congress in the [*6] exercise of the patent power may not overreach the restraints imposed by the stated constitutional purpose. Nor may it enlarge the patent monopoly without regard to the innovation, advancement or social benefit gained thereby. Moreover, Congress may not authorize the issuance of patents whose effects are to remove existent knowledge from the public domain, or to restrict free access to materials already available. Innovation, advancement, and things which add to the sum of useful knowledge are inherent requisites in a patent system which by constitutional command must "promote the Progress of . . . useful Arts." This is the *standard* expressed in the Constitution and it may not be ignored. And it is in this light that patent validity "requires reference to a standard written into the Constitution." *A. & P. Tea Co. v. Supermarket Corp., supra, at 154* (concurring opinion).

1 The provision appears in the Constitution spliced together with the copyright provision, which we omit as not relevant here. See H. R. Rep. No. 1923, 82d Cong., 2d Sess., at 4 (1952); DeWolf, An Outline of Copyright Law, p. 15 (Boston, 1925).

[***LEdHR5] [5] [***LEdHR6] [6] [***LEdHR7] [7]Within the limits of the constitutional grant, the Congress may, of course, implement the stated purpose of the Framers by selecting the policy which in its judgment best effectuates the constitutional aim. This is but a corollary to the grant to Congress of any Article I power. *Gibbons v. Ogden, 9 Wheat. 1.* Within the scope established by the Constitution, Congress may set out conditions and tests for patentability. *McClurg v. Kingsland, 1 How. 202, 206.* It is the duty of the Commissioner of Patents and of the courts in the administration of the patent system to give effect to the constitutional standard by appropriate application, in each case, of the statutory scheme of the Congress.

Congress quickly responded to the bidding of the Constitution by enacting the Patent Act of 1790 during the second session of the First Congress. It created an agency in the Department of State headed by the Secretary of State, the Secretary of the Department of War [*7] and the Attorney General, any two of whom could issue a patent for a period not exceeding 14 years to any petitioner that "hath . . . invented or discovered any useful art, manufacture, . . . or device, or any improvement therein not before known or used" if the board found that "the invention or discovery [was] sufficiently useful and important . . . ." 1 Stat. 110. This group, whose members administered the patent system along with their other public duties, was known by its own designation as "Commissioners for the Promotion of Useful Arts."

Thomas Jefferson, who as Secretary of State was a member of the group, was its moving spirit and might well be called the "first administrator of our patent system." See Federico, Operation of the Patent Act of 1790, 18 J. Pat. Off. Soc. 237, 238 (1936). He was not only an [***551] administrator of the patent system under the 1790 Act, but was also the author of the 1793 Patent Act. In addition, Jefferson was himself an inventor of great note. His unpatented improvements on plows, to mention but one line of his inventions, won acclaim and recognition on both sides of the Atlantic. Because of his active interest and influence in the early development of the patent system, Jefferson's views on the general nature of the limited patent monopoly under the Constitution, as well as his conclusions as to conditions for patentability under the statutory scheme, are worthy of note.

Jefferson, like other Americans, had an instinctive aversion to monopolies. It was a monopoly on tea that sparked the Revolution and Jefferson certainly did not favor an equivalent form of monopoly under the new government. His abhorrence of monopoly extended initially to patents as well. From France, he wrote to Madison (July 1788) urging a *Bill of Rights* provision restricting monopoly, and as against the argument that [*8] limited [**689] monopoly might serve to incite "ingenuity," he argued forcefully that "the benefit even of limited monopolies is too doubtful to be opposed to that of their general suppression," V Writings of Thomas Jefferson, at 47 (Ford ed., 1895).

His views ripened, however, and in another letter to Madison (Aug. 1789) after the drafting of the *Bill of*

Rights, Jefferson stated that he would have been pleased by an express provision in this form:

"Art. 9. Monopolies may be allowed to persons for their own productions in literature & their own inventions in the arts, for a term not exceeding -- years but for no longer term & no other purpose." *Id., at 113.*

And he later wrote:

"Certainly an inventor ought to be allowed a right to the benefit of his invention for some certain time. . . . Nobody wishes more than I do that ingenuity should receive a liberal encouragement." Letter to Oliver Evans (May 1807), V Writings of Thomas Jefferson, at 75-76 (Washington ed.).

Jefferson's philosophy on the nature and purpose of the patent monopoly is expressed in a letter to Isaac McPherson (Aug. 1813), a portion of which we set out in the margin. [2] He rejected a natural-rights [***552] theory in [*9] intellectual property rights and clearly recognized the social and economic rationale of the patent system. The patent monopoly was not designed to secure to the inventor his natural right in his discoveries. Rather, it was a reward, an inducement, to bring forth new knowledge. The grant of an exclusive right to an invention was the creation of society -- at odds with the inherent free nature of disclosed ideas -- and was not to be freely given. Only inventions and discoveries which furthered human knowledge, and were new and useful, justified the special inducement of a limited private monopoly. Jefferson did not believe in granting patents for small details, obvious improvements, or frivolous devices. His writings evidence his insistence upon a high level of patentability.

2 "Stable ownership is the gift of social law, and is given late in the progress of society. It would be curious then, if an idea, the fugitive fermentation of an individual brain, could, of natural right, be claimed in exclusive and stable property. If nature has made any one thing less susceptible than all others of exclusive property, it is the action of the thinking power called an idea, which an individual may exclusively possess as long as he keeps it to himself; but the moment it is divulged, it forces itself into the possession of every one, and the receiver cannot dispossess himself of it. Its peculiar character, too, is that no one possesses the less, because every other possesses the whole of it. He who receives an idea from me, receives instruction himself without lessening mine; as he who lights his taper at mine, receives light without darkening me. That ideas should freely spread from one to another over the globe, for the moral and mutual instruction of man, and improvement of his condition, seems to have been peculiarly and benevolently designed by nature, when she made them, like fire, expansible over all space, without lessening their density in any point, and like the air in which we breathe, move, and have our physical being, incapable of confinement or exclusive appropriation. Inventions then cannot, in nature, be a subject of property. Society may give an exclusive right to the profits arising from them, as an encouragement to men to pursue ideas which may produce utility, but this may or may not be done, according to the will and convenience of the society, without claim or complaint from any body." VI Writings of Thomas Jefferson, at 180-181 (Washington ed.).

As a member of the patent board for several years, Jefferson saw clearly the difficulty in "drawing a line between the things which are worth to the public the embarrassment of an exclusive patent, and those which are not." The board on which he served sought to draw such a line and formulated several rules which [**690] are preserved in [*10] Jefferson's correspondence. [3] Despite the board's efforts, Jefferson saw "with what slow progress a system of general rules could be matured." Because of the "abundance" of cases and the fact that the investigations occupied "more time of the members of the board than they could spare from higher duties, the whole was turned over to the judiciary, to be matured into a system, under which every one might know when his actions were safe and lawful." Letter to McPherson, *supra*, at 181, 182. Apparently Congress agreed with Jefferson and the board that the courts should develop additional conditions for patentability. Although the Patent Act was amended, revised or codified some 50 times between 1790 and 1950, Congress steered clear of a statutory set of requirements other than the bare novelty and utility tests reformulated in Jefferson's draft of the 1793 Patent Act.

3 "[A] machine of which we are possessed, might be applied by every man to any use of which it is susceptible." Letter to Isaac McPherson, *supra*, at 181.

"[A] change of material should not give title to a patent. As the making a ploughshare of cast rather than of wrought iron; a comb of iron instead of horn or of ivory . . . ." *Ibid.*

"[A] mere change of form should give no right to a patent, as a high-quartered shoe instead of a low one; a round hat instead of a three-square; or a square bucket instead of a round one." *Id., at 181-182.*

> "[A combined use of old implements.] A man has a right to use a saw, an axe, a plane separately; may he not combine their uses on the same piece of wood?" Letter to Oliver Evans (Jan. 1814), VI Writings of Thomas Jefferson, at 298 (Washington ed.).

III.

The difficulty of formulating conditions for patentability was heightened by the generality of the constitutional grant and the statutes implementing it, together with the underlying policy of the patent system that "the things which are worth to the public the embarrassment [*11] of an exclusive patent," as Jefferson put it, must outweigh the restrictive effect of the limited patent monopoly. The inherent problem was to develop some means of weeding out those inventions which would not be disclosed or devised but for the inducement of a patent.

[***553] This Court formulated a general condition of patentability in 1851 in *Hotchkiss v. Greenwood, 11 How. 248*. The patent involved a mere substitution of materials -- porcelain or clay for wood or metal in doorknobs -- and the Court condemned it, holding: [4]

"Unless more ingenuity and skill . . . were required . . . than were possessed by an ordinary mechanic acquainted with the business, there was an absence of that degree of skill and ingenuity which constitute essential elements of every invention. In other words, the improvement is the work of the skilful mechanic, not that of the inventor." At p. 267.

> 4 In historical retrospect, the specific result in *Hotchkiss* flows directly from an application of one of the rules of the original board of "Commissioners," n. 3, second rule, *supra*.

Hotchkiss, by positing the condition that a patentable invention evidence more ingenuity and skill than that possessed by an ordinary mechanic acquainted with the business, merely distinguished between new and useful innovations that were capable of sustaining a patent and those that were not. The *Hotchkiss* test laid the cornerstone of the judicial evolution suggested by Jefferson and left to the courts by Congress. The language in the case, and in those which followed, gave birth to "invention" as a word of legal art signifying patentable inventions. Yet, as this Court has observed, "the truth is the word ['invention'] cannot be defined in such manner as [**691] to afford any substantial aid in determining whether a particular device involves an exercise of the inventive faculty [*12] or not." *McClain v. Ortmayer, 141 U.S. 419, 427 (1891)*; *A. & P. Tea Co. v. Supermarket Corp., supra, at 151*. Its use as a label brought about a large variety of opinions as to its meaning both in the Patent Office, in the courts, and at the bar. The *Hotchkiss* formulation, however, lies not in any label, but in its functional approach to questions of patentability. In practice, *Hotchkiss* has required a comparison between the subject matter of the patent, or patent application, and the background skill of the calling. It has been from this comparison that patentability was in each case determined.

IV.

The 1952 Patent Act.

[***LEdHR8] [8]The Act sets out the conditions of patentability in three sections. An analysis of the structure of these three sections indicates that patentability is dependent upon three explicit conditions: novelty and utility as articulated and defined in *§ 101* and *§ 102*, and nonobviousness, the new statutory formulation, as set out in *§ 103*. The first two sections, which trace closely the 1874 codification, express the "new and useful" tests which have always existed in the statutory scheme and, for our purposes here, need no clarification. [5] The pivotal [*13] section around which the present [***554] controversy centers is *§ 103*. It provides:

"*§ 103. Conditions for patentability; non-obvious subject matter*

[HN1] "A patent may not be obtained though the invention is not identically disclosed or described as set forth in *section 102* of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention [**692] was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made."

> 5 "*§ 101. Inventions patentable*
>
> "Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title."
>
> "*§ 102. Conditions for patentability; novelty and loss of right to patent*
>
> "A person shall be entitled to a patent unless --
>
> "(a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country,

before the invention thereof by the applicant for patent, or

"(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of the application for patent in the United States, or

"(c) he has abandoned the invention, or

"(d) the invention was first patented or caused to be patented by the applicant or his legal representatives or assigns in a foreign country prior to the date of the application for patent in this country on an application filed more than twelve months before the filing of the application in the United States, or

"(e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or

"(f) he did not himself invent the subject matter sought to be patented, or

"(g) before the applicant's invention thereof the invention was made in this country by another who had not abandoned, suppressed, or concealed it. In determining priority of invention there shall be considered not only the respective dates of conception and reduction to practice of the invention, but also the reasonable diligence of one who was first to conceive and last to reduce to practice, from a time prior to conception by the other."

The precursors of these sections are to be found in the Act of February 21, 1793, c. 11, 1 Stat. 318; Act of July 4, 1836, c. 357, 5 Stat. 117; Act of July 8, 1870, c. 230, 16 Stat. 198; Rev. Stat. § 4886 (1874).

[*14] The section is cast in relatively unambiguous terms. [HN2] Patentability is to depend, in addition to novelty and utility, upon the "non-obvious" nature of the "subject matter sought to be patented" to a person having ordinary skill in the pertinent art.

The first sentence of this section is strongly reminiscent of the language in *Hotchkiss*. Both formulations place emphasis on the pertinent art existing at the time the invention was made and both are implicitly tied to advances in that art. The major distinction is that Congress has emphasized "nonobviousness" as the operative test of the section, rather than the less definite "invention" language of *Hotchkiss* that Congress thought had led to "a large variety" of expressions in decisions and writings. In the title itself the Congress used the phrase "Conditions for patentability; *non-obvious subject matter*" (italics added), thus focusing upon "nonobviousness" rather than "invention." 6 The Senate and House Reports, S. Rep. No. 1979, 82d Cong., 2d Sess. (1952); H. R. Rep. No. 1923, 82d Cong., 2d Sess. (1952), reflect this emphasis in these terms:

"*Section 103*, for the first time in our statute, provides a condition which exists in the law and has existed for more than 100 years, but only by reason of decisions of the courts. An invention which has been made, and which is new in the sense that the same thing has not been made before, may still not be patentable if the difference between the new thing and what was known before is not considered sufficiently great to warrant a patent. That has [***555] been expressed in a large variety of ways in decisions of [*15] the courts and in writings. *Section 103* states this requirement in the title. It refers to the difference between the subject matter sought to be patented and the prior art, meaning what was known before as described in *section 102*. If this difference is such that the subject matter as a whole would have been obvious at the time to a person skilled in the art, then the subject matter cannot be patented.

"That provision paraphrases language which has often been used in decisions of the courts, and the section is added to the statute for uniformity and definiteness. This section should have a stabilizing effect and minimize great departures which have appeared in some cases." H. R. Rep., *supra*, at 7; S. Rep., *supra*, at 6.

6 The corresponding provision in the preliminary draft was titled "Conditions for patentability, *lack of invention*" (italics added), Proposed Revision and Amendment of the Patent Laws, Preliminary Draft with Notes, House Committee on the Judiciary (Committee Print, 1950).

[***LEdHR9] [9]It is undisputed that this section was, for the first time, a statutory expression of an additional requirement for patentability, originally expressed in *Hotchkiss*. It also seems apparent that Congress intended by the last sentence of *§ 103* to abolish the test it believed this Court announced in the controversial phrase "flash of creative genius," used in *Cuno Corp. v. Automatic Devices Corp., 314 U.S. 84 (1941)*.[7]

7 The sentence in which the phrase occurs reads: "The new device, however useful it may be, must reveal the flash of creative genius, not merely the skill of the calling." At p. 91. Although some writers and lower courts found in the language connotations as to the frame of mind of the inventors, none were so intended. The

opinion approved *Hotchkiss* specifically, and the reference to "flash of creative genius" was but a rhetorical embellishment of language going back to 1833. Cf. "exercise of genius," *Shaw* v. *Cooper*, 7 Pet. 292; "inventive genius," *Reckendorfer v. Faber, 92 U.S. 347 (1876)*; *Concrete Appliances Co. v. Gomery, 269 U.S. 177*; "flash of thought," *Densmore v. Scofield, 102 U.S. 375 (1880)*; "intuitive genius," *Potts v. Creager, 155 U.S. 597 (1895)*. Rather than establishing a more exacting standard, *Cuno* merely rhetorically restated the requirement that the subject matter sought to be patented must be beyond the skill of the calling. It was the device, not the invention, that had to reveal the "flash of creative genius." See Boyajian, The Flash of Creative Genius, An Alternative Interpretation, 25 J. Pat. Off. Soc. 776, 780, 781 (1943); *Pacific Contact Laboratories, Inc. v. Solex Laboratories, Inc., 209 F.2d 529, 533*; *Brown & Sharpe Mfg. Co. v. Kar Engineering Co., 154 F.2d 48, 51-52*; *In re Shortell, 31 C. C. P. A. (Pat.) 1062, 1069, 142 F.2d 292, 295-296.*

[*16] It [**693] is contended, however, by some of the parties and by several of the *amici* that the first sentence of *§ 103* was intended to sweep away judicial precedents and to lower the level of patentability. Others contend that the Congress intended to codify the essential purpose reflected in existing judicial precedents -- the rejection of insignificant variations and innovations of a commonplace sort -- and also to focus inquiries under *§ 103* upon nonobviousness, rather than upon "invention," as a means of achieving more stability and predictability in determining patentability and validity.

The Reviser's Note to this section, [8] with apparent reference to *Hotchkiss*, recognizes that judicial [***556] requirements as to "lack of patentable novelty [have] been followed since at least as early as 1850." The note indicates that the section was inserted because it "may have some stabilizing effect, and also to serve as a basis for the addition at a later time of some criteria which may be worked out." To this same effect are the reports of both Houses, *supra*, which state that the first sentence [*17] of the section "paraphrases language which has often been used in decisions of the courts, and the section is added to the statute for uniformity and definiteness."

8 "There is no provision corresponding to the first sentence explicitly stated in the present statutes, but the refusal of patents by the Patent Office, and the holding of patents invalid by the courts, on the ground of lack of invention or lack of patentable novelty has been followed since at least as early as 1850. This paragraph is added with the view that an explicit statement in the statute may have some stabilizing effect, and also to serve as a basis for the addition at a later time of some criteria which may be worked out.

"The second sentence states that patentability as to this requirement is not to be negatived by the manner in which the invention was made, that is, it is immaterial whether it resulted from long toil and experimentation or from a flash of genius."

[***LEdHR1B] [1B] [***LEdHR2B] [2B]

We believe that this legislative history, as well as other sources, [9] shows that the revision was not intended by Congress to change the general level of patentable invention. We conclude that the section was intended merely as a codification of judicial precedents embracing the *Hotchkiss* condition, with congressional directions that inquiries into the obviousness of the subject matter sought to be patented are a prerequisite to patentability.

9 See Efforts to Establish a Statutory Standard of Invention, Study No. 7, Senate Subcommittee on Patents, Trademarks, and Copyrights, 85th Cong., 1st Sess. (Committee Print, 1958); Hearings, Subcommittee No. 3, House Committee on the Judiciary, on H. R. 3760, 82d Cong., 1st Sess. (1951).

V.

[***LEdHR10] [10]Approached in this light, the *§ 103* additional condition, when followed realistically, will permit a more practical test of patentability. The emphasis on nonobviousness is one of inquiry, not [**694] quality, and, as such, comports with the constitutional strictures.

[***LEdHR11] [11][HN3] While the ultimate question of patent validity is one of law, *A. & P. Tea Co. v. Supermarket Corp., supra, at 155*, the *§ 103* condition, which is but one of three conditions, each of which must be satisfied, lends itself to several basic factual inquiries. Under *§ 103*, the scope and content of the prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background, the obviousness or nonobviousness of the subject matter is determined. Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances [*18] surrounding the origin of the subject matter sought to be patented. As indicia of obviousness or nonobviousness, these inquiries may have relevancy. See Note, Subtests of "Nonobviousness": A

Nontechnical Approach to Patent Validity, 112 U. Pa. L. Rev. 1169 (1964).

[***LEdHR12] [12]This is not to say, however, that there will not be difficulties in applying the nonobviousness test. What is obvious is not a question upon which there is likely to be uniformity of thought in every given factual context. The difficulties, however, are comparable to those encountered daily by the courts in such frames of reference as negligence and scienter, and should be amenable to a case-by-case development. We believe that strict observance of the requirements laid down here will result in that uniformity and definiteness which Congress called for in the 1952 Act.

[***LEdHR13] [13]While we have focused attention on the appropriate standard to be applied by the courts, it must be remembered that the primary responsibility [***557] for sifting out unpatentable material lies in the Patent Office. To await litigation is -- for all practical purposes -- to debilitate the patent system. We have observed a notorious difference between the standards applied by the Patent Office and by the courts. While many reasons can be adduced to explain the discrepancy, one may well be the free rein often exercised by Examiners in their use of the concept of "invention." In this connection we note that the Patent Office is confronted with a most difficult task. Almost 100,000 applications for patents are filed each year. Of these, about 50,000 are granted and the backlog now runs well over 200,000. 1965 Annual Report of the Commissioner of Patents 13-14. This is itself a compelling reason for the Commissioner to strictly adhere to the 1952 Act as interpreted here. This would, we believe, not only expedite disposition but [*19] bring about a closer concurrence between administrative and judicial precedent. [10]

10 The President has appointed a Commission on the Patent System. Executive Order No. 11215, 30 Fed. Reg. 4661 (April 10, 1965). It is hoped that its studies may develop more efficient administrative procedures and techniques that will further expedite dispositions and at the same time insure the strict application of appropriate tests of patentability.

[HN4] Although we conclude here that the inquiry which the Patent Office and the courts must make as to patentability must be beamed with greater intensity on the requirements of *§ 103*, it bears repeating that we find no change in the general strictness with which the overall test is to be applied. We have been urged to find in *§ 103* a relaxed standard, supposedly a congressional reaction to the "increased standard" applied by this Court in its decisions over the last 20 or 30 years. The standard has remained invariable in this Court. Technology, however, has advanced -- and with remarkable rapidity in the last 50 years. Moreover, the ambit of applicable art in given fields of science has widened by [**695] disciplines unheard of a half century ago. It is but an evenhanded application to require that those persons granted the benefit of a patent monopoly be charged with an awareness of these changed conditions. The same is true of the less technical, but still useful arts. He who seeks to build a better mousetrap today has a long path to tread before reaching the Patent Office.

VI.

We now turn to the application of the conditions found necessary for patentability to the cases involved here:

A. *The Patent in Issue in No. 11*, Graham *v.* John Deere Co.

This patent, No. 2,627,798 (hereinafter called the '798 patent) relates to a spring clamp which permits plow shanks to be pushed upward when they hit obstructions [*20] in the soil, and then springs the shanks back into normal position when the obstruction is passed over. The device, which we show diagrammatically in the accompanying sketches (Appendix, Fig. 1), is fixed to the plow frame as a unit. The mechanism around which the controversy centers is basically a hinge. The top half of it, known as the upper plate (marked 1 in the sketches), is a heavy metal piece clamped to the plow frame (2) and is stationary relative to the plow frame. The lower half of the hinge, known as the hinge plate (3), is connected to the rear of the upper plate by a [***558] hinge pin (4) and rotates downward with respect to it. The shank (5), which is bolted to the forward end of the hinge plate (at 6), runs beneath the plate and parallel to it for about nine inches, passes through a stirrup (7), and then continues backward for several feet curving down toward the ground. The chisel (8), which does the actual plowing, is attached to the rear end of the shank. As the plow frame is pulled forward, the chisel rips through the soil, thereby plowing it. In the normal position, the hinge plate and the shank are kept tight against the upper plate by a spring (9), which is atop the upper plate. A rod (10) runs through the center of the spring, extending down through holes in both plates and the shank. Its upper end is bolted to the top of the spring while its lower end is hooked against the underside of the shank.

When the chisel hits a rock or other obstruction in the soil, the obstruction forces the chisel and the rear portion of the shank to move upward. The shank is pivoted (at 11) against the rear of the hinge plate and pries open the hinge against the closing tendency of the spring. (See sketch labeled "Open Position," Appendix, Fig. 1.) This closing tendency is caused by the fact that, as the hinge is opened, the connecting rod is pulled downward and the spring is compressed. When the obstruction

[*21] is passed over, the upward force on the chisel disappears and the spring pulls the shank and hinge plate back into their original position. The lower, rear portion of the hinge plate is constructed in the form of a stirrup (7) which brackets the shank, passing around and beneath it. The shank fits loosely into the stirrup (permitting a slight up and down play). The stirrup is designed to prevent the shank from recoiling away from the hinge plate, and thus prevents excessive strain on the shank near its bolted connection. The stirrup also girds the shank, preventing it from fishtailing from side to side.

In practical use, a number of spring-hinge-shank combinations are clamped to a plow frame, forming a set of ground-working chisels capable of withstanding the shock of rocks and other obstructions in the soil without breaking the shanks.

Background of the Patent.

Chisel plows, as they are called, were developed for plowing in areas where the ground is relatively free from rocks or [**696] stones. Originally, the shanks were rigidly attached to the plow frames. When such plows were used in the rocky, glacial soils of some of the Northern States, they were found to have serious defects. As the chisels hit buried rocks, a vibratory motion was set up and tremendous forces were transmitted to the shank near its connection to the frame. The shanks would break. Graham, one of the petitioners, sought to meet that problem, and in 1950 obtained a patent, U.S. No. 2,493,811 (hereinafter '811), on a spring clamp which solved some of the difficulties. Graham and his companies manufactured and sold the '811 clamps. In 1950, Graham modified the '811 structure and filed for a patent. That patent, the one in issue, was granted in 1953. This suit against competing plow manufacturers resulted from charges by petitioners that several of respondents' devices infringed the '798 patent.

[*22] *The Prior Art.*

Five prior patents indicating the state of the art were cited by the Patent Office in the prosecution of the '798 application. Four of these [***559] patents, 10 other United States patents and two prior-use spring-clamp arrangements not of record in the '798 file wrapper were relied upon by respondents as revealing the prior art. The District Court and the Court of Appeals found that the prior art "as a whole in one form or another contains all of the mechanical elements of the 798 Patent." One of the prior-use clamp devices not before the Patent Examiner -- Glencoe -- was found to have "all of the elements."

We confine our discussion to the prior patent of Graham, '811, and to the Glencoe clamp device, both among the references asserted by respondents. The Graham '811 and '798 patent devices are similar in all elements, save two: (1) the stirrup and the bolted connection of the shank to the hinge plate do not appear in '811; and (2) the position of the shank is reversed, being placed in patent '811 above the hinge plate, sandwiched between it and the upper plate. The shank is held in place by the spring rod which is hooked against the bottom of the hinge plate passing through a slot in the shank. Other differences are of no consequence to our examination. In practice the '811 patent arrangement permitted the shank to wobble or fishtail because it was not rigidly fixed to the hinge plate; moreover, as the hinge plate was below the shank, the latter caused wear on the upper plate, a member difficult to repair or replace.

Graham's '798 patent application contained 12 claims. All were rejected as not distinguished from the Graham '811 patent. The inverted position of the shank was specifically rejected as was the bolting of the shank to the hinge plate. The Patent Office examiner found these to be "matters of design well within the expected skill of [*23] the art and devoid of invention." Graham withdrew the original claims and substituted the two new ones which are substantially those in issue here. His contention was that wear was reduced in patent '798 between the shank and the heel or rear of the upper plate. " He also emphasized several new features, the relevant one here being that the bolt used to connect the hinge plate and shank maintained the upper face of the shank in continuing [**697] and constant contact with the underface of the hinge plate.

> 11 In '811, where the shank was above the hinge plate, an upward movement of the chisel forced the shank up against the underside of the rear of the upper plate. The upper plate thus provided the fulcrum about which the hinge was pried open. Because of this, as well as the location of the hinge pin, the shank rubbed against the heel of the upper plate causing wear both to the plate and to the shank. By relocating the hinge pin and by placing the hinge plate between the shank and the upper plate, as in '798, the rubbing was eliminated and the wear point was changed to the hinge plate, a member more easily removed or replaced for repair.

Graham did not urge before the Patent Office the greater "flexing" qualities of the '798 patent arrangement which he so heavily relied on in the courts. The sole element in patent '798 which petitioners argue before us is the interchanging of the shank and hinge plate and the consequences flowing from this arrangement. The contention is that this arrangement -- which petitioners claim is not disclosed in the prior art -- permits the shank to flex under stress for its *entire* length. As we have

sketched (see sketch, "Graham '798 Patent" in Appendix, Fig. 2), when the chisel hits an obstruction the resultant force (A) pushes the rear of the shank upward and the [***560] shank pivots against the rear of the hinge plate at (C). The natural tendency is for that portion of the shank between the pivot point and the bolted connection (*i. e.*, between C and D) to bow downward and away from the hinge plate. The maximum distance [*24] (B) that the shank moves away from the plate is slight -- for emphasis, greatly exaggerated in the sketches. This is so because of the strength of the shank and the short -- nine inches or so -- length of that portion of the shank between (C) and (D). On the contrary, in patent '811 (see sketch, "Graham '811 Patent" in Appendix, Fig. 2), the pivot point is the upper plate at point (c); and while the tendency for the shank to bow between points (c) and (d) is the same as in '798, the shank is restricted because of the underlying hinge plate and cannot flex as freely. In practical effect, the shank flexes only between points (a) and (c), and not along the entire length of the shank, as in '798. Petitioners say that this difference in flex, though small, effectively absorbs the tremendous forces of the shock of obstructions whereas prior art arrangements failed.

The Obviousness of the Differences.

We cannot agree with petitioners. We assume that the prior art does not disclose such an arrangement as petitioners claim in patent '798. Still we do not believe that the argument on which petitioners' contention is bottomed supports the validity of the patent. The tendency of the shank to flex is the same in all cases. If free-flexing, as petitioners now argue, is the crucial difference above the prior art, then it appears evident that the desired result would be obtainable by not boxing the shank within the confines of the hinge. [12] The only other effective place available in the arrangement was to attach it below the hinge plate and run it through a [*25] stirrup or bracket that would not disturb its flexing qualities. Certainly a person having ordinary skill in the prior art, given the fact that the flex in the shank could be utilized more effectively if allowed to run the entire length of the shank, would immediately see that the thing to do was what Graham did, *i. e.*, invert the shank and the hinge plate.

> 12 Even petitioners' expert testified to that effect:
>
> "Q. Given the same length of the forward portion of the clamp . . . you would anticipate that the magnitude of flex [in '798] would be precisely the same or substantially the same as in 811, wouldn't you?
>
> "A. I would think so."

Petitioners' argument basing validity on the free-flex theory raised for the first time on appeal is reminiscent of *Lincoln Engineering Co. v. Stewart-Warner Corp., 303 U.S. 545 (1938)*, where the Court called such an effort "an afterthought. No such function . . . is hinted at in the specifications of the patent. If this were so vital an element in the functioning of the apparatus it is strange that all mention of it was omitted." At p. 550. No "flexing" argument [**698] was raised in the Patent Office. Indeed, the trial judge specifically found that "flexing is not a claim of the patent in suit . . ." and would not permit interrogation as to flexing in the accused devices. Moreover, the clear testimony of petitioners' experts shows that the flexing advantages flowing from the '798 arrangement are not, in fact, a significant feature in the patent. [13]

> 13 "Q. . . . Do you regard the small degree of flex in the forward end of the shank that lies between the pivot point and the point of spring attachment to be of any significance or any importance to the functioning of a device such as 798? A. Unless you are approaching the elastic limit, I think this flexing will reduce the maximum stress at the point of pivot there, where the maximum stress does occur. I think it will reduce that. I don't know how much.
>
> "Q. Do you think it is a substantial factor, a factor of importance in the functioning of the structure? A. Not a great factor, no."
>
> The same expert previously testified similarly in *Jeoffroy Mfg., Inc. v. Graham, 219 F.2d 511.*

[***LEdHR14] [14]We [***561] find no nonobvious facets in the '798 arrangement. The wear and repair claims were sufficient to overcome [*26] the patent examiner's original conclusions as to the validity of the patent. However, some of the prior art, notably Glencoe, was not before him. There the hinge plate is below the shank but, as the courts below found, all of the elements in the '798 patent are present in the Glencoe structure. Furthermore, even though the position of the shank and hinge plate appears reversed in Glencoe, the mechanical operation is identical. The shank there pivots about the underside of the stirrup, which in Glencoe is *above* the shank. In other words, the stirrup in Glencoe serves exactly the same function as the heel of the hinge plate in '798. The mere shifting of the wear point to the heel of the '798 hinge plate from the stirrup of Glencoe -- itself a part of the hinge plate -- presents no operative mechanical distinctions, much less nonobvious differences.

B. *The Patent in Issue in No. 37*, Calmar, Inc. *v.* Cook Chemical Co., *and in No. 43*, Colgate-Palmolive Co. *v.* Cook Chemical Co.

The single patent [14] involved in these cases relates to a plastic finger sprayer with a "hold-down" lid used as a built-in dispenser for containers or bottles packaging liquid products, principally household insecticides. Only the first two of the four claims in the patent are involved here and we, therefore, limit our discussion to them. We do not set out those claims here since they are printed in *220 F.Supp., at 417-418.*

14 The patent is U.S. No. 2,870,943 issued in 1959 to Cook Chemical Co. as assignee of Baxter I. Scoggin, Jr., the inventor. In No. 37, Calmar is the manufacturer of an alleged infringing device, and, in No. 43, Colgate is a customer of Calmar and user of its device.

In essence the device here combines a finger-operated pump sprayer, mounted in a container or bottle by means of a container cap, with a plastic overcap which screws over the top of and depresses the sprayer (see Appendix, [*27] Fig. 3). The pump sprayer passes through the container cap and extends down into the liquid in the container; the overcap fits over the pump sprayer and screws down on the outside of a collar mounting or retainer which is molded around the body of the sprayer. When the overcap is screwed down on this collar mounting a seal is formed by the engagement of a circular ridge or rib located above the threads on the collar mounting with a mating shoulder located inside the overcap above its threads. [15] The overcap, as it is screwed down, depresses the pump plunger rendering the pump inoperable and when the seal is effected, [**699] any liquid which might seep into the overcap through or around the pump is prevented from leaking out of the overcap. [***562] The overcap serves also to protect the sprayer head and prevent damage to it during shipment or merchandising. When the overcap is in place it does not reach the cap of the container or bottle and in no way engages it since a slight space is left between those two pieces.

15 Our discussion here relates to the overcap seal. The container itself is sealed in the customary way through the use of a container gasket located between the container and the container cap.

The device, called a shipper-sprayer in the industry, is sold as an integrated unit with the overcap in place enabling the insecticide manufacturer to install it on the container or bottle of liquid in a single operation in an automated bottling process. The ultimate consumer simply unscrews and discards the overcap, the pump plunger springs up and the sprayer is ready for use.

The Background of the Patent.

For many years manufacturers engaged in the insecticide business had faced a serious problem in developing sprayers that could be integrated with the containers or bottles in which the insecticides were marketed. Originally, insecticides were applied through the use of tin [*28] sprayers, not supplied by the manufacturer. In 1947, Cook Chemical, an insecticide manufacturer, began to furnish its customers with plastic pump dispensers purchased from Calmar. The dispenser was an unpatented finger-operated device mounted in a perforated cardboard holder and hung over the neck of the bottle or container. It was necessary for the ultimate consumer to remove the cap of the container and insert and attach the sprayer to the latter for use.

Hanging the sprayer on the side of the container or bottle was both expensive and troublesome. Packaging for shipment had to be a hand operation, and breakage and pilferage as well as the loss of the sprayer during shipment and retail display often occurred. Cook Chemical urged Calmar to develop an integrated sprayer that could be mounted directly in a container or bottle during the automated filling process and that would not leak during shipment or retail handling. Calmar did develop some such devices but for various reasons they were not completely successful. The situation was aggravated in 1954 by the entry of Colgate-Palmolive into the insecticide trade with its product marketed in aerosol spray cans. These containers, which used compressed gas as a propellent to dispense the liquid, did not require pump sprayers.

During the same year Calmar was acquired by the Drackett Company. Cook Chemical became apprehensive of its source of supply for pump sprayers and decided to manufacture its own through a subsidiary, Bakan Plastics, Inc. Initially, it copied its design from the unpatented Calmar sprayer, but an officer of Cook Chemical, Scoggin, was assigned to develop a more efficient device. By 1956 Scoggin had perfected the shipper-sprayer in suit and a patent was granted in 1959 to Cook Chemical as his assignee. In the interim Cook Chemical began to use Scoggin's device and also marketed [*29] it to the trade. The device was well received and soon became widely used.

In the meanwhile, Calmar employed two engineers, Corsette and Cooprider, to perfect a shipper-sprayer and by 1958 it began to market its SS-40, a device very much similar to Scoggin's. When the Scoggin patent issued, Cook Chemical charged Calmar's SS-40 with infringement and this suit followed.

The Opinions of the District Court and the Court of Appeals.

At the outset it is well to point up [***563] that the parties have always disagreed as to the scope and definition of the invention claimed in the patent in suit. Cook Chemical contends that the invention encompasses a unique combination of admittedly old elements and that patentability is found in the result produced. Its expert testified that the invention was "the first commercially successful, inexpensive integrated shipping closure pump unit which permitted automated assembly with a container of household [**700] insecticide or similar liquids to produce a practical, ready-to-use package which could be shipped without external leakage and which was so organized that the pump unit with its hold-down cap could be itself assembled and sealed and then later assembled and sealed on the container without breaking the first seal." Cook Chemical stresses the long-felt need in the industry for such a device; the inability of others to produce it; and its commercial success -- all of which, contends Cook, evidences the nonobvious nature of the device at the time it was developed. On the other hand, Calmar says that the differences between Scoggin's shipper-sprayer and the prior art relate only to the design of the overcap and that the differences are so inconsequential that the device as a whole would have been obvious at the time of its invention to a person having ordinary skill in the art.

[*30] Both courts accepted Cook Chemical's contentions. While the exact basis of the District Court's holding is uncertain, the court did find the subject matter of the patent new, useful and nonobvious. It concluded that Scoggin "had produced a sealed and protected sprayer unit which the manufacturer need only screw onto the top of its container in much the same fashion as a simple metal cap." *220 F.Supp., at 418*. Its decision seems to be bottomed on the finding that the Scoggin sprayer solved the long-standing problem that had confronted the industry. [16] The Court of Appeals also found validity in the "novel 'marriage' of the sprayer with the insecticide container" which took years in discovery and in "the immediate commercial success" which it enjoyed. While finding that the individual elements of the invention were "not novel per se" the court found "nothing in the prior art suggesting Scoggin's unique combination of these old features . . . as would solve the . . . problems which for years beset the insecticide industry." It concluded that "the . . . [device] meets the exacting standard required for a combination of old elements to rise to the level of patentable invention by fulfilling the long-felt need with an economical, efficient, utilitarian apparatus which achieved novel results and immediate commercial success." *336 F.2d, at 114*.

16 "By the same reasoning, may it not also be said that if [the device] solved a long-sought need, it was likewise novel? If it meets the requirements of being new, novel and useful, it was the subject of invention, although it may have been a short step, nevertheless it was the last step that ended the journey. The last step is the one that wins and he who takes it when others could not, is entitled to patent protection." *220 F.Supp., at 421*.

The Prior Art.

Only two of the five prior art patents cited by the Patent Office Examiner in the prosecution of Scoggin's application are necessary to our discussion, *i. e.*, Lohse [*31] *U.S. Patent No. 2,119,884* (1938) and Mellon *U.S. Patent No. 2,586,687* (1952). Others are cited by Calmar that were not before the Examiner, but of [***564] these our purposes require discussion of only the Livingstone *U.S. Patent No. 2,715,480* (1953). Simplified drawings of each of these patents are reproduced in the Appendix, Figs. 4-6, for comparison and description.

The Lohse patent (Fig. 4) is a shipper-sprayer designed to perform the same function as Scoggin's device. The differences, recognized by the District Court, are found in the overcap seal which in Lohse is formed by the skirt of the overcap engaging a washer or gasket which rests upon the upper surface of the container cap. The court emphasized that in Lohse "there are no seals above the threads and below the sprayer head." *220 F.Supp., at 419*.

The Mellon patent (Fig. 5), however, discloses the idea of effecting a seal above the threads of the overcap. Mellon's device, likewise a shipper-sprayer, differs from Scoggin's in that its overcap [**701] screws directly on the container, and a gasket, rather than a rib, is used to effect the seal.

[***LEdHR15A] [15A]Finally, Livingstone (Fig. 6) shows a seal above the threads accomplished without the use of a gasket or washer. [17] Although Livingstone's arrangement was designed to cover and protect pouring spouts, his sealing feature is strikingly similar to Scoggin's. Livingstone uses a tongue and groove technique in which the tongue, located on the upper surface of the collar, fits into a groove on the inside of the overcap. Scoggin employed the rib and shoulder seal in the identical position and with less efficiency because the Livingstone technique is inherently a more stable structure, forming an interlock that withstands distortion of the overcap when subjected to rough handling. Indeed, Cook Chemical has now incorporated the Livingstone closure into its own shipper-sprayers as had Calmar in its SS-40.

[***LEdHR15B] [15B]

> 17 While the sealing feature was not specifically claimed in the Livingstone patent, it was disclosed in the drawings and specifications. Under long-settled law the feature became public property. *Miller v. Brass Co., 104 U.S. 350, 352 (1882).*

The Invalidity of the Patent.

Let us first return to the fundamental disagreement between the parties. Cook Chemical, as we noted at the outset, urges that the invention must be viewed as the overall combination, or -- putting it in the language of the statute -- that we must consider the subject matter sought to be patented taken as a whole. With this position, taken in the abstract, there is, of course, no quibble. But the history of the prosecution of the Scoggin application in the Patent Office reveals a substantial divergence in respondent's present position.

As originally submitted, the Scoggin application contained 15 claims which in very broad terms claimed the entire combination of spray pump and overcap. No mention of, or claim for, the sealing features was made. All 15 claims were rejected by the Examiner because (1) the applicant was vague and indefinite as to what the invention was, and (2) the claims were met by Lohse. Scoggin canceled these claims and submitted new ones. Upon a further series of rejections and new submissions, the Patent Office Examiner, after an office interview, at last relented. It is crystal clear that after the first rejection, Scoggin relied entirely upon the sealing arrangement as the exclusive patentable difference in his combination. It is likewise clear that it was [***565] on that feature that the Examiner allowed the claims. In fact, in a letter accompanying the final submission of claims, Scoggin, through his attorney, stated that "agreement was reached between the Honorable Examiner and applicant's attorney relative to *limitations* which must be in the claims in [*33] order to define novelty over the previously applied disclosure of Lohse when considered in view of the newly cited patents of Mellon and Darley, Jr." (Italics added.)

Moreover, those limitations were specifically spelled out as (1) the use of a rib seal and (2) an overcap whose lower edge did not contact the container cap. Mellon was distinguished, as was the Darley patent, *infra*, n. 18, on the basis that although it disclosed a hold-down cap with a seal located above the threads, it did not disclose a rib seal disposed in such position as to cause the lower peripheral edge of the overcap "to be maintained out of contacting relationship with [the container] cap . . . when . . . [the overcap] was screwed [on] tightly . . . ." Scoggin maintained that the "obvious modification" of Lohse in view of Mellon would be merely to place the Lohse gasket above the threads with the lower edge of the overcap remaining in tight contact with the container cap or neck of the container itself. In other words, the [**702] Scoggin invention was limited to the use of a rib -- rather than a washer or gasket -- and the existence of a slight space between the overcap and the container cap.

[***LEdHR16] [16] [***LEdHR17] [17]It is, of course, well settled that [HN5] an invention is construed not only in the light of the claims, but also with reference to the file wrapper or prosecution history in the Patent Office. *Hogg v. Emerson, 11 How. 587 (1850)*; *Crawford v. Heysinger, 123 U.S. 589 (1887).* Claims as allowed must be read and interpreted with reference to rejected ones and to the state of the prior art; and claims that have been narrowed in order to obtain the issuance of a patent by distinguishing the prior art cannot be sustained to cover that which was previously by limitation eliminated from the patent. *Powers-Kennedy Co. v. Concrete Co., 282 U.S. 175, 185-186 (1930)*; *Schriber Co. v. Cleveland Trust Co., 311 U.S. 211, 220-221 (1940).*

[*34] Here, the patentee obtained his patent only by accepting the limitations imposed by the Examiner. The claims were carefully drafted to reflect these limitations and Cook Chemical is not now free to assert a broader view of Scoggin's invention. The subject matter as a whole reduces, then, to the distinguishing features clearly incorporated into the claims. We now turn to those features.

As to the space between the skirt of the overcap and the container cap, the District Court found:

"Certainly without a space so described, there could be no inner seal within the cap, but such a space is not new or novel, but it is necessary to the formation of the seal within the hold-down cap.

"*To me this language is descriptive of an element of the patent but not a part of the invention.* It is too simple, really, to require much discussion. In this device the hold-down cap was intended to perform two functions -- to hold down the sprayer head and to form a solid tight seal between the shoulder and the collar below. In assembling the element it is necessary to provide [***566] this space in order to form the seal." *220 F.Supp., at 420.* (Italics added.)

The court correctly viewed the significance of that feature. We are at a loss to explain the Examiner's allowance on the basis of such a distinction. Scoggin was able to convince the Examiner that Mellon's cap contacted the bottle neck while his did not. Although the drawings included in the Mellon application show that the cap might touch the neck of the bottle when fully

screwed down, there is nothing -- absolutely nothing -- which indicates that the cap was designed at any time to *engage* the bottle neck. It is palpably evident that Mellon embodies a seal formed by a gasket compressed [*35] between the cap and the bottle neck. It follows that the cap in Mellon will not seal if it does not bear down on the gasket and this would be impractical, if not impossible, under the construction urged by Scoggin before the Examiner. Moreover, the space so strongly asserted by Cook Chemical appears quite plainly on the Livingstone device, a reference not cited by the Examiner.

[***LEdHR18] [18]The substitution of a rib built into a collar likewise presents no patentable difference above the prior art. It was fully disclosed and dedicated to the public in the Livingstone patent. Cook Chemical argues, however, that Livingstone is not in the *pertinent* prior art because it relates to liquid containers having pouring spouts rather than pump sprayers. Apart from the fact that respondent made no such objection to similar [**703] references cited by the Examiner, [18] so restricted a view of the applicable prior art is not justified. The problems confronting Scoggin and the insecticide industry were not insecticide problems; they were mechanical closure problems. Closure devices in such a closely related art as pouring spouts for liquid containers are at the very least pertinent references. See, II Walker on Patents § 260 (Deller ed. 1937).

18 In addition to Livingstone and Mellon, the Examiner cited Slade, *U.S. Patent No. 2,844,290* (hold-down cap for detergent cans having a pouring spout); Nilson, *U.S. Patent No. 2,118,222* (combined cap and spout for liquid dispensing containers); Darley, Jr., *U.S. Patent No. 1,447,712* (containers for toothpaste, cold creams and other semi-liquid substances).

[***LEdHR19] [19]Cook Chemical insists, however, that the development of a workable shipper-sprayer eluded Calmar, who had long and unsuccessfully sought to solve the problem. And, further, that the long-felt need in the industry for a device such as Scoggin's together with its wide commercial success supports its patentability. These legal inferences [*36] or subtests do focus attention on economic and motivational rather than technical issues and are, therefore, more susceptible of judicial treatment than are the highly technical facts often present in patent litigation. See Judge Learned Hand in *Reiner v. I. Leon Co., 285 F.2d 501, 504 (1960).* See also Note, Subtests of "Nonobviousness": A Nontechnical Approach to Patent Validity, 112 U. Pa. L. Rev. 1169 (1964). Such inquiries may lend a helping hand to the judiciary which, as Mr. Justice Frankfurter observed, is most ill-fitted to discharge the technological duties cast upon it by patent legislation. *Marconi Wireless Co. v. United States, 320 U.S. 1, 60 (1943).* They may also serve to "guard against slipping into use of hindsight," *Monroe Auto Equipment Co. v. Heckethorn Mfg. & Sup. Co., 332 F.2d 406, 412 (1964)*, and to resist the temptation to read into the prior art the teachings of the invention in issue.

[***567] [***LEdHR20] [20] [***LEdHR21] [21]However, these factors do not, in the circumstances of this case, tip the scales of patentability. The Scoggin invention, as limited by the Patent Office and accepted by Scoggin, rests upon exceedingly small and quite nontechnical mechanical differences in a device which was old in the art. At the latest, those differences were rendered apparent in 1953 by the appearance of the Livingstone patent, and unsuccessful attempts to reach a solution to the problems confronting Scoggin made before that time became wholly irrelevant. It is also irrelevant that no one apparently chose to avail himself of knowledge stored in the Patent Office and readily available by the simple expedient of conducting a patent search -- a prudent and nowadays common preliminary to well organized research. *Mast, Foos & Co. v. Stover Mfg. Co., 177 U.S. 485 (1900).* To us, the limited claims of the Scoggin patent are clearly evident from the prior art as it stood at the time of the invention.

[*37] [***LEdHR22] [22]We conclude that the claims in issue in the Scoggin patent must fall as not meeting the test of *§ 103*, since the differences between them and the pertinent prior art would have been obvious to a person reasonably skilled in that art.

The judgment of the Court of Appeals in No. 11 is affirmed. The judgment of the Court of Appeals in Nos. 37 and 43 is reversed and the cases remanded to the District Court for disposition not inconsistent with this opinion.

It is so ordered.

MR. JUSTICE STEWART took no part in the consideration or decision of Nos. 37 and 43.

MR. JUSTICE FORTAS took no part in the consideration or decision of these cases.

[SEE ILLUSTRATION IN ORIGINAL.]

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U.S. Appln. No. 10/770,639
Reference No. 13

LEXSEE 127 S. CT. 1727

KSR INTERNATIONAL CO., Petitioner v. TELEFLEX INC. et al.

No. 04-1350

SUPREME COURT OF THE UNITED STATES

127 S. Ct. 1727; 167 L. Ed. 2d 705; 2007 U.S. LEXIS 4745; 75 U.S.L.W. 4289; 82 U.S.P.Q.2D (BNA) 1385; 20 Fla. L. Weekly Fed. S 248

November 28, 2006, Argued
April 30, 2007, Decided

NOTICE:

[***1] The LEXIS pagination of this document is subject to change pending release of the final published version.

SUBSEQUENT HISTORY: On remand at *Teleflex, Inc. v. KSR Int'l Co., 228 Fed. Appx. 988, 2007 U.S. App. LEXIS 16051 (Fed. Cir., June 20, 2007)*

PRIOR HISTORY: ON WRIT OF CERTIORARI TO THE UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT.
Teleflex, Inc. v. KSR Int'l Co., 119 Fed. Appx. 282, 2005 U.S. App. LEXIS 176 (Fed. Cir., 2005)

DISPOSITION: Reversed and remanded.

CASE SUMMARY:

PROCEDURAL POSTURE: Respondent, licensees of a patent, alleged that petitioner, a competitor, infringed the licensees' patent for an accelerator pedal assembly for vehicles, but the competitor asserted that the patent claim in dispute was invalid as obvious under *35 U.S.C.S. § 103*. Upon the grant of a writ of certiorari, the competitor appealed the judgment of the U.S. Court of Appeals for the Federal Circuit which reversed a summary judgment of patent invalidity.

OVERVIEW: To satisfy customer needs, the competitor modified its design for an adjustable pedal system for vehicles with cable-actuated throttles by adding a modular sensor to make the system compatible with vehicles using computer-controlled throttles. The licensees contended that the competitor infringed the patent claim of a position-adjustable pedal assembly with an electronic pedal position sensor attached a fixed pivot point. The U.S. Supreme Court unanimously held that the patent claim was invalid as obvious since mounting an available sensor on a fixed pivot point of the competitor's pedal was a design step well within the grasp of a person of ordinary skill in the relevant art, and the benefit of doing so was obvious. The marketplace created a strong incentive to convert mechanical pedals to electronic pedals, and the prior art taught a number of methods for doing so. Further, the problem to be solved by the patent claim did not limit its application as prior art, the competitor's showing that it was obvious to try a combination of elements sufficiently supported the finding of obviousness, and the claim was the result of ordinary skill and common sense rather than innovation.

OUTCOME: The judgment reversing the summary judgment of invalidity was reversed, and the case was remanded for further proceedings.

LexisNexis(R) Headnotes

Patent Law > Nonobviousness > General Overview
[HN1] *35 U.S.C.S. § 103* forbids issuance of a patent when the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
Patent Law > Nonobviousness > Elements & Tests > Secondary Considerations
[HN2] Under *35 U.S.C.S. § 103*, the scope and content of prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved.

Against this background the obviousness or nonobviousness of the subject matter is determined. Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented. While the sequence of these questions might be reordered in any particular case, the factors continue to define the inquiry that controls. If a court, or patent examiner, conducts this analysis and concludes the claimed subject matter was obvious, the claim is invalid under *§ 103*.

Patent Law > Infringement Actions > Defenses > Patent Invalidity > Validity Presumption
[HN3] By direction of *35 U.S.C.S. § 282*, an issued patent is presumed valid.

Patent Law > Nonobviousness > Elements & Tests > Predictability
[HN4] A patent for a combination which only unites old elements with no change in their respective functions obviously withdraws what is already known into the field of its monopoly and diminishes the resources available to skillful men. This is a principal reason for declining to allow patents for what is obvious. The combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results.

Patent Law > Nonobviousness > Elements & Tests > Predictability
[HN5] When a work is available in one field of endeavor, design incentives and other market forces can prompt variations of it, either in the same field or a different one. If a person of ordinary skill can implement a predictable variation, *35 U.S.C.S. § 103* likely bars its patentability. For the same reason, if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond his or her skill. A court must ask whether the improvement is more than the predictable use of prior art elements according to their established functions.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN6] Rejection of a patent on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support a legal conclusion of obviousness. However, the analysis need not seek out precise teachings directed to the specific subject matter of the challenged claim, for a court can take account of the inferences and creative steps that a person of ordinary skill in the art would employ.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN7] A patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art. Although common sense directs one to look with care at a patent application that claims as innovation the combination of two known devices according to their established functions, it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does. This is so because inventions in most, if not all, instances rely upon building blocks long since uncovered, and claimed discoveries almost of necessity will be combinations of what, in some sense, is already known.

Patent Law > Nonobviousness > Elements & Tests > Secondary Considerations
[HN8] The obviousness analysis in the patent context cannot be confined by a formalistic conception of the words teaching, suggestion, and motivation, or by overemphasis on the importance of published articles and the explicit content of issued patents. The diversity of inventive pursuits and of modern technology counsels against limiting the analysis in this way. In many fields it may be that there is little discussion of obvious techniques or combinations, and it often may be the case that market demand, rather than scientific literature, will drive design trends. Granting patent protection to advances that would occur in the ordinary course without real innovation retards progress and may, in the case of patents combining previously known elements, deprive prior inventions of their value or utility.

Patent Law > Nonobviousness > Elements & Tests > Manner of Conception
Patent Law > Nonobviousness > Elements & Tests > Predictability
[HN9] In determining whether the subject matter of a patent claim is obvious, neither the particular motivation nor the avowed purpose of the patentee controls. What matters is the objective reach of the claim. If the claim extends to what is obvious, it is invalid under *35 U.S.C.S. § 103*. One of the ways in which a patent's subject matter can be proved obvious is by noting that there existed at

the time of invention a known problem for which there was an obvious solution encompassed by the patent's claims.

Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
[HN10] A problem motivating a patentee may be only one of many addressed by the patent's subject matter. The question is not whether the combination was obvious to the patentee but whether the combination was obvious to a person with ordinary skill in the art.

Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
[HN11] When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product not of innovation but of ordinary skill and common sense. In that instance the fact that a combination was obvious to try might show that it was obvious under *35 U.S.C.S. § 103*.

Patent Law > Nonobviousness > Elements & Tests > Hindsight
[HN12] In a patent obviousness case, a factfinder must be aware of the distortion caused by hindsight bias and must be cautious of arguments reliant upon ex post reasoning. Rigid preventative rules that deny factfinders recourse to common sense, however, are neither necessary under U.S. Supreme Court case law nor consistent with it.

Patent Law > Infringement Actions > Summary Judgment > General Overview
Patent Law > Nonobviousness > Elements & Tests > General Overview
Patent Law > Nonobviousness > Evidence & Procedure > Fact & Law Issues
[HN13] In considering summary judgment on a question of patent obviousness, a district court can and should take into account expert testimony, which may resolve or keep open certain questions of fact. That is not the end of the issue, however. The ultimate judgment of obviousness is a legal determination. Where the content of the prior art, the scope of the patent claim, and the level of ordinary skill in the art are not in material dispute, and the obviousness of the claim is apparent in light of these factors, summary judgment is appropriate.

Constitutional Law > Congressional Duties & Powers > Copyright & Patent Clause
Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN14] As progress beginning from higher levels of achievement is expected in the normal course, the results of ordinary innovation are not the subject of exclusive rights under the patent laws. Were it otherwise patents might stifle, rather than promote, the progress of useful arts. *U.S. Const. art. I, § 8, cl. 8*. These premises lead to the bar on patents claiming obvious subject matter established by case law and codified in *35 U.S.C.S. § 103*. Application of the bar must not be confined within a test or formulation too constrained to serve its purpose.

DECISION:

[**705] Company that added modular sensor to its automobile-accelerator-pedal system held entitled to summary judgment in infringement action by holder of license for patent covering assembly with electronic sensor, as pertinent claim was "obvious" within meaning of *35 U.S.C.S. § 103*.

SUMMARY:

Procedural posture: Respondent, licensees of a patent, alleged that petitioner, a competitor, infringed the licensees' patent for an accelerator pedal assembly for vehicles, but the competitor asserted that the patent claim in dispute was invalid as obvious under *35 U.S.C.S. § 103*. Upon the grant of a writ of certiorari, the competitor appealed the judgment of the U.S. Court of Appeals for the Federal Circuit which reversed a summary judgment of patent invalidity.

Overview: To satisfy customer needs, the competitor modified its design for an adjustable pedal system for vehicles with cable-actuated throttles by adding a modular sensor to make the system compatible with vehicles using computer-controlled throttles. The licensees contended that the competitor infringed the patent claim of a position-adjustable pedal assembly with an electronic pedal position sensor attached a fixed pivot point. The U.S. Supreme Court unanimously held that the patent claim was invalid as obvious since mounting an available sensor on a fixed pivot point of the competitor's pedal was a design step well within the grasp of a person of ordinary skill in the relevant art, and the benefit of doing so was obvious. The marketplace created a strong incentive to convert mechanical pedals to electronic pedals, and the prior art taught a number of methods for doing so. Further, the problem to be solved by the patent claim did not limit its application as prior art, the competitor's showing that it was obvious to try a combination of elements [**706] sufficiently supported the finding of ob-

viousness, and the claim was the result of ordinary skill and common sense rather than innovation.

Outcome: The judgment reversing the summary judgment of invalidity was reversed, and the case was remanded for further proceedings.

LAWYERS' EDITION HEADNOTES:

[**LEdHN1]

PATENTS § 19.1

PATENTABILITY -- OBVIOUSNESS OF SUBJECT MATTER

Headnote:[1]

35 U.S.C.S. § 103 forbids issuance of a patent when the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains.

[**LEdHN2]

PATENTS § 19 PATENTS § 19.1

PATENTABILITY -- MECHANICAL SKILL -- OBVIOUSNESS OF SUBJECT MATTER

Headnote:[2]

Under *35 U.S.C.S. § 103*, the scope and content of prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background the obviousness or nonobviousness of the subject matter is determined. Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented. While the sequence of these questions might be reordered in any particular case, the factors continue to define the inquiry that controls. If a court, or patent examiner, conducts this analysis and concludes the claimed subject matter was obvious, the claim is invalid under *§ 103*.

[**LEdHN3]

EVIDENCE § 333

PATENT -- PRESUMPTION OF VALIDITY

Headnote:[3]

By direction of *35 U.S.C.S. § 282*, an issued patent is presumed valid.

[**LEdHN4]

PATENTS § 37

PATENTABILITY -- COMBINATION OF OLD ELEMENTS

Headnote:[4]

A patent for a combination which only unites old elements with no change in their respective functions obviously withdraws what is already known into the field of its monopoly and diminishes the resources available to skillful men. This is a principal reason for declining to allow patents for what is obvious. The combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results.

[**LEdHN5]

PATENTS § 50

PATENTABILITY -- OBVIOUSNESS OF IMPROVEMENT

Headnote:[5]

When a work is available in one field of endeavor, design incentives and other market forces can prompt variations of it, either in the same field or a different one. If a person of ordinary skill can implement a predictable variation, *35 U.S.C.S. § 103* likely bars its patentability. For the same reason, if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond his or her skill. A court must ask whether the [**707] improvement is more than the predictable use of prior art elements according to their established functions.

[**LEdHN6]

PATENTS § 19.1

PATENTABILITY -- OBVIOUSNESS OF SUBJECT MATTER

Headnote:[6]

Rejection of a patent on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support a legal conclusion of obviousness. However, the analysis need not seek out precise teachings directed to the specific subject matter of the challenged claim, for a court can take account of the inferences and creative steps that a person of ordinary skill in the art would employ.

[**LEdHN7]

PATENTS § 19.1

PATENTABILITY -- COMPOSITION OF ELEMENTS -- OBVIOUSNESS

Headnote:[7]

A patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art. Although common sense directs one to look with care at a patent application that claims as innovation the combination of two known devices according to their established functions, it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does. This is so because inventions in most, if not all, instances rely upon building blocks long since uncovered, and claimed discoveries almost of necessity will be combinations of what, in some sense, is already known.

[**LEdHN8]

PATENTS § 19.1

PATENTABILITY -- OBVIOUSNESS OF TECHNIQUES OR COMBINATIONS -- SCIENTIFIC LITERATURE

Headnote:[8]

The obviousness analysis in the patent context cannot be confined by a formalistic conception of the words teaching, suggestion, and motivation, or by overemphasis on the importance of published articles and the explicit content of issued patents. The diversity of inventive pursuits and of modern technology counsels against limiting the analysis in this way. In many fields it may be that there is little discussion of obvious techniques or combinations, and it often may be the case that market demand, rather than scientific literature, will drive design trends. Granting patent protection to advances that would occur in the ordinary course without real innovation retards progress and may, in the case of patents combining previously known elements, deprive prior inventions of their value or utility.

[**LEdHN9]

PATENTS § 19.1

PATENTABILITY -- SUBJECT MATTER -- DETERMINATION WHETHER OBVIOUS

Headnote:[9]

In determining whether the subject matter of a patent claim is obvious, neither the particular motivation nor the avowed purpose of the patentee controls. What matters is the objective reach of the claim. If the claim extends to what is obvious, it is invalid under *35 U.S.C.S. § 103*. One of the ways in which a patent's subject matter can be proved obvious is by noting that there existed at the time of invention a known problem for which there was an obvious solution encompassed by the patent's claims.

[**LEdHN10]

PATENTS § 19.1

PATENTABILITY -- OBVIOUSNESS

Headnote:[10]

A problem motivating a patentee may be only one of many addressed by the patent's subject matter. The question is not whether the combination was obvious to the patentee [**708] but whether the combination was obvious to a person with ordinary skill in the art.

[**LEdHN11]

PATENTS § 19 PATENTS § 19.1

PATENTABILITY -- ORDINARY SKILL -- OBVIOUSNESS

Headnote:[11]

When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product not of innovation but of ordinary skill and common sense. In that instance the fact that a combination was obvious to try might show that it was obvious under *35 U.S.C.S. § 103*.

[**LEdHN12]

PATENTS § 19.1

PATENTABILITY -- OBVIOUSNESS

Headnote:[12]

In a patent obviousness case, a factfinder must be aware of the distortion caused by hindsight bias and must be cautious of arguments reliant upon ex post reasoning. Rigid preventative rules that deny factfinders recourse to common sense, however, are neither necessary under U.S. Supreme Court case law nor consistent with it.

[**LEdHN13]

SUMMARY JUDGMENT AND JUDGMENT ON PLEADINGS § 5

PATENTABILITY -- OBVIOUSNESS OF CLAIM -- SUMMARY JUDGMENT

Headnote:[13]

In considering summary judgment on a question of patent obviousness, a district court can and should take into account expert testimony, which may resolve or keep open certain questions of fact. That is not the end of the issue, however. The ultimate judgment of obviousness is a legal determination. Where the content of the prior art, the scope of the patent claim, and the level of ordinary skill in the art are not in material dispute, and the obviousness of the claim is apparent in light of these factors, summary judgment is appropriate.

[**LEdHN14]

PATENTS § 17 PATENTS § 19.1

PATENTABILITY -- ORDINARY INNOVATION -- OBVIOUS SUBJECT MATTER

Headnote:[14]

As progress beginning from higher levels of achievement is expected in the normal course, the results of ordinary innovation are not the subject of exclusive rights under the patent laws. Were it otherwise patents might stifle, rather than promote, the progress of useful arts. *U.S. Const. art. I, § 8, cl. 8.* These premises lead to the bar on patents claiming obvious subject matter established by case law and codified in *35 U.S.C.S. § 103.* Application of the bar must not be confined within a test or formulation too constrained to serve its purpose.

SYLLABUS

[**709] To control a conventional automobile's speed, the driver depresses or releases the gas pedal, which interacts with the throttle via a cable or other mechanical link. Because the pedal's position in the footwell normally cannot be adjusted, a driver wishing to be closer or farther from it must either reposition himself in the seat or move the seat, both of which can be imperfect solutions for smaller drivers in cars with deep footwells. This prompted inventors to design and patent pedals that could be adjusted to change their locations. The Asano patent reveals a support structure whereby, when the pedal location is [***2] adjusted, one of the pedal's pivot points stays fixed. Asano is also designed so that the force necessary to depress the pedal is the same regardless of location adjustments. The Redding patent reveals a different, sliding mechanism where both the pedal and the pivot point are adjusted.

In newer cars, computer-controlled throttles do not operate through force transferred from the pedal by a mechanical link, but open and close valves in response to electronic signals. For the computer to know what is happening with the pedal, an electronic sensor must translate the mechanical operation into digital data. Inventors had obtained a number of patents for such sensors. The so-called *'936 patent* taught that it was preferable to detect the pedal's position in the pedal mechanism, not in the engine, so the patent disclosed a pedal with an electronic sensor on a pivot point in the pedal assembly. The Smith patent taught that to prevent the wires connecting the sensor to the computer from chafing and wearing out, the sensor should be put on a fixed part of the pedal assembly rather than in or on the pedal's footpad. Inventors had also patented self-contained modular sensors, which can be [***3] taken off the shelf and attached to any mechanical pedal to allow it to function with a computer-controlled throttle. The *'068 patent* disclosed one such sensor. Chevrolet also manufactured trucks using modular sensors attached to the pedal support bracket, adjacent to the pedal and engaged [**710] with the pivot shaft about which the pedal rotates. Other patents disclose electronic sensors attached to adjustable pedal assemblies. For example, the Rixon patent locates the sensor in the pedal footpad, but is known for wire chafing.

After petitioner KSR developed an adjustable pedal system for cars with cable-actuated throttles and obtained its *'976 patent* for the design, General Motors Corporation (GMC) chose KSR to supply adjustable pedal systems for trucks using computer-controlled throttles. To make the *'976* pedal compatible with the trucks, KSR added a modular sensor to its design. Respondents (Teleflex) hold the exclusive license for the Engelgau patent, claim 4 of which discloses a position-adjustable pedal assembly with an electronic pedal position sensor attached a fixed pivot point. Despite having denied a similar, broader claim, the U. S. Patent and Trademark Office (PTO) had allowed [***4] claim 4 because it included the limitation of a fixed pivot position, which distinguished the design from Redding's. Asano was neither included among the Engelgau patent's prior art references nor mentioned in the patent's prosecution, and the PTO did not have before it an adjustable pedal with a fixed pivot point. After learning of KSR's design for GMC, Teleflex sued for infringement, asserting that KSR's pedal system infringed the Engelgau patent's claim 4. KSR countered that claim 4 was invalid under *§ 103* of the Patent Act, which forbids issuance of a patent when "the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art."

Graham v. John Deere Co. of Kansas City, 383 U.S. 1, 17-18, 86 S. Ct. 684, 15 L. Ed. 2d 545, set out an ob-

jective analysis for applying *§ 103*: "[T]he scope and content of the prior art are . . . determined; differences between the prior art and the claims at issue are . . . ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background the obviousness or nonobviousness [***5] of the subject matter is determined. Such secondary considerations as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented." While the sequence of these questions might be reordered in any particular case, the factors define the controlling inquiry. However, seeking to resolve the obviousness question with more uniformity and consistency, the Federal Circuit has employed a "teaching, suggestion, or motivation" (TSM) test, under which a patent claim is only proved obvious if the prior art, the problem's nature, or the knowledge of a person having ordinary skill in the art reveals some motivation or suggestion to combine the prior art teachings.

The District Court granted KSR summary judgment. After reviewing pedal design history, the Engelgau patent's scope, and the relevant prior art, the court considered claim 4's validity, applying *Graham*'s framework to determine whether under summary-judgment standards KSR had demonstrated that claim 4 was obvious. The court found "little difference" between the prior art's teachings and claim 4: [***6] Asano taught everything contained in the claim except [**711] using a sensor to detect the pedal's position and transmit it to a computer controlling the throttle. That additional aspect was revealed in, *e.g.*, the *'068 patent* and Chevrolet's sensors. The court then held that KSR satisfied the TSM test, reasoning (1) the state of the industry would lead inevitably to combinations of electronic sensors and adjustable pedals, (2) Rixon provided the basis for these developments, and (3) Smith taught a solution to Rixon's chafing problems by positioning the sensor on the pedal's fixed structure, which could lead to the combination of a pedal like Asano with a pedal position sensor.

Reversing, the Federal Circuit ruled the District Court had not applied the TSM test strictly enough, having failed to make findings as to the specific understanding or principle within a skilled artisan's knowledge that would have motivated one with no knowledge of the invention to attach an electronic control to the Asano assembly's support bracket. The Court of Appeals held that the District Court's recourse to the nature of the problem to be solved was insufficient because, unless the prior art references [***7] addressed the precise problem that the patentee was trying to solve, the problem would not motivate an inventor to look at those references. The appeals court found that the Asano pedal was designed to ensure that the force required to depress the pedal is the same no matter how the pedal is adjusted, whereas Engelgau sought to provide a simpler, smaller, cheaper adjustable electronic pedal. The Rixon pedal, said the court, suffered from chafing but was not designed to solve that problem and taught nothing helpful to Engelgau's purpose. Smith, in turn, did not relate to adjustable pedals and did not necessarily go to the issue of motivation to attach the electronic control on the pedal assembly's support bracket. So interpreted, the court held, the patents would not have led a person of ordinary skill to put a sensor on an Asano-like pedal. That it might have been obvious to try that combination was likewise irrelevant. Finally, the court held that genuine issues of material fact precluded summary judgment.

Held:

The Federal Circuit addressed the obviousness question in a narrow, rigid manner that is inconsistent with *§ 103* and this Court's precedents. KSR provided convincing [***8] evidence that mounting an available sensor on a fixed pivot point of the Asano pedal was a design step well within the grasp of a person of ordinary skill in the relevant art and that the benefit of doing so would be obvious. Its arguments, and the record, demonstrate that the Engelgau patent's claim 4 is obvious. Pp. 11-24.

1. *Graham* provided an expansive and flexible approach to the obviousness question that is inconsistent with the way the Federal Circuit applied its TSM test here. Neither *§ 103*'s enactment nor *Graham*'s analysis disturbed the Court's earlier instructions concerning the need for caution in granting a patent based on the combination of elements found in the prior art. See *Great Atlantic & Pacific Tea Co. v. Supermarket Equipment Corp., 340 U.S. 147, 152, 71 S. Ct. 127, 95 L. Ed. 162, 1951 Dec. Comm'r Pat. 572* Such a combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results. See, *e.g., United States v. Adams, 383 U.S. 39, 50-52, 86 S. Ct. 708, 15 L. Ed. 2d 572, 174 Ct. Cl. 1293* When a work is available in one field, design incentives and other market forces [**712] can prompt variations of it, either in the same field or in another. If a person [***9] of ordinary skill in the art can implement a predictable variation, and would see the benefit of doing so, *§ 103* likely bars its patentability. Moreover, if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond that person's skill. A court must ask whether the improvement is more than the predictable use of prior-art elements according to their established functions. Following these principles may be difficult if the claimed subject matter involves more than the simple substitution of one known element for another or the mere applica-

tion of a known technique to a piece of prior art ready for the improvement. To determine whether there was an apparent reason to combine the known elements in the way a patent claims, it will often be necessary to look to interrelated teachings of multiple patents; to the effects of demands known to the design community or present in the marketplace; and to the background knowledge possessed by a person having ordinary skill in the art. To facilitate review, this analysis should [***10] be made explicit. But it need not seek out precise teachings directed to the challenged claim's specific subject matter, for a court can consider the inferences and creative steps a person of ordinary skill in the art would employ. Pp. 11-14.

(b) The TSM test captures a helpful insight: A patent composed of several elements is not proved obvious merely by demonstrating that each element was, independently, known in the prior art. Although common sense directs caution as to a patent application claiming as innovation the combination of two known devices according to their established functions, it can be important to identify a reason that would have prompted a person of ordinary skill in the art to combine the elements as the new invention does. Inventions usually rely upon building blocks long since uncovered, and claimed discoveries almost necessarily will be combinations of what, in some sense, is already known. Helpful insights, however, need not become rigid and mandatory formulas. If it is so applied, the TSM test is incompatible with this Court's precedents. The diversity of inventive pursuits and of modern technology counsels against confining the obviousness analysis [***11] by a formalistic conception of the words teaching, suggestion, and motivation, or by overemphasizing the importance of published articles and the explicit content of issued patents. In many fields there may be little discussion of obvious techniques or combinations, and market demand, rather than scientific literature, may often drive design trends. Granting patent protection to advances that would occur in the ordinary course without real innovation retards progress and may, for patents combining previously known elements, deprive prior inventions of their value or utility. Since the TSM test was devised, the Federal Circuit doubtless has applied it in accord with these principles in many cases. There is no necessary inconsistency between the test and the *Graham* analysis. But a court errs where, as here, it transforms general principle into a rigid rule limiting the obviousness inquiry. Pp. 14-15.

(c) The flaws in the Federal Circuit's analysis relate mostly to its [**713] narrow conception of the obviousness inquiry consequent in its application of the TSM test. The Circuit first erred in holding that courts and patent examiners should look only to the problem the patentee was trying [***12] to solve. Under the correct analysis, any need or problem known in the field and addressed by the patent can provide a reason for combining the elements in the manner claimed. Second, the appeals court erred in assuming that a person of ordinary skill in the art attempting to solve a problem will be led only to those prior art elements designed to solve the same problem. The court wrongly concluded that because Asano's primary purpose was solving the constant ratio problem, an inventor considering how to put a sensor on an adjustable pedal would have no reason to consider putting it on the Asano pedal. It is common sense that familiar items may have obvious uses beyond their primary purposes, and a person of ordinary skill often will be able to fit the teachings of multiple patents together like pieces of a puzzle. Regardless of Asano's primary purpose, it provided an obvious example of an adjustable pedal with a fixed pivot point, and the prior art was replete with patents indicating that such a point was an ideal mount for a sensor. Third, the court erred in concluding that a patent claim cannot be proved obvious merely by showing that the combination of elements was obvious to try. [***13] When there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill in the art has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product not of innovation but of ordinary skill and common sense. Finally, the court drew the wrong conclusion from the risk of courts and patent examiners falling prey to hindsight bias. Rigid preventative rules that deny recourse to common sense are neither necessary under, nor consistent with, this Court's case law. Pp. 15-18.

2. Application of the foregoing standards demonstrates that claim 4 is obvious. Pp. 18-23.

(a) The Court rejects Teleflex's argument that the Asano pivot mechanism's design prevents its combination with a sensor in the manner claim 4 describes. This argument was not raised before the District Court, and it is unclear whether it was raised before the Federal Circuit. Given the significance of the District Court's finding that combining Asano with a pivot-mounted pedal position sensor fell within claim 4's scope, it is apparent that Teleflex would [***14] have made clearer challenges if it intended to preserve this claim. Its failure to clearly raise the argument, and the appeals court's silence on the issue, lead this Court to accept the District Court's conclusion. Pp. 18-20.

(b) The District Court correctly concluded that when Engelgau designed the claim 4 subject matter, it was obvious to a person of ordinary skill in the art to combine Asano with a pivot-mounted pedal position sensor. There then was a marketplace creating a strong incentive to convert mechanical pedals to electronic pedals, and the

prior art taught a number of methods for doing so. The Federal Circuit considered the issue too narrowly by, in effect, asking whether a pedal designer writing on a blank slate would have chosen both Asano and a modular sensor similar to the ones used in the Chevrolet trucks and [**714] disclosed in the *'068 patent*. The proper question was whether a pedal designer of ordinary skill in the art, facing the wide range of needs created by developments in the field, would have seen an obvious benefit to upgrading Asano with a sensor. For such a designer starting with Asano, the question was where to attach the sensor. The *'936 patent* taught [***15] the utility of putting the sensor on the pedal device. Smith, in turn, explained not to put the sensor on the pedal footpad, but instead on the structure. And from Rixon's known wire-chafing problems, and Smith's teaching that the pedal assemblies must not precipitate any motion in the connecting wires, the designer would know to place the sensor on a nonmoving part of the pedal structure. The most obvious such point is a pivot point. The designer, accordingly, would follow Smith in mounting the sensor there. Just as it was possible to begin with the objective to upgrade Asano to work with a computer-controlled throttle, so too was it possible to take an adjustable electronic pedal like Rixon and seek an improvement that would avoid the wire-chafing problem. Teleflex has not shown anything in the prior art that taught away from the use of Asano, nor any secondary factors to dislodge the determination that claim 4 is obvious. Pp. 20-23.

3. The Court disagrees with the Federal Circuit's holding that genuine issues of material fact precluded summary judgment. The ultimate judgment of obviousness is a legal determination. *Graham, 383 U.S., at 17, 86 S. Ct. 684, 15 L. Ed. 2d 545*. Where, as here, the [***16] prior art's content, the patent claim's scope, and the level of ordinary skill in the art are not in material dispute and the claim's obviousness is apparent, summary judgment is appropriate. P. 23.

119 Fed. Appx. 282, reversed and remanded.

COUNSEL: James W. Dabney argued the cause for petitioner.

Thomas G. Hungar argued the cause for the United States, as amicus curiae, by special leave of court.

Thomas C. Goldstein argued the cause for respondents.

JUDGES: Kennedy, J., delivered the opinion for a unanimous Court.

OPINION BY: KENNEDY

OPINION

[*1734] Justice **Kennedy** delivered the opinion of the Court.

Teleflex Incorporated and its subsidiary Technology Holding Company--both referred to here as Teleflex--sued KSR International Company for patent infringement. The patent at issue, *United States Patent No. 6,237,565* B1, is entitled "Adjustable Pedal Assembly With Electronic Throttle Control." Supplemental App. 1. The patentee is Steven J. Engelgau, and the patent is referred to as "the Engelgau patent." Teleflex holds the exclusive license to the patent.

Claim 4 of the Engelgau patent describes a mechanism for combining an electronic sensor with an adjustable automobile pedal so the pedal's position can be transmitted to a computer that controls the throttle in the vehicle's engine. When Teleflex accused KSR of infringing the Engelgau patent by adding an electronic sensor to one of KSR's previously [***17] designed pedals, KSR countered that claim 4 was invalid under the Patent Act, *35 U.S.C. § 103*, because its subject matter was obvious.

[HN1] [**LEdHR1] [1]*Section 103* forbids issuance of a patent when "the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having [**715] ordinary skill in the art to which said subject matter pertains."

In *Graham v. John Deere Co. of Kansas City, 383 U.S. 1, 86 S. Ct. 684, 15 L. Ed. 2d 545 (1966)*, the Court set out a framework for applying the statutory language of *§ 103*, language itself based on the logic of the earlier decision in *Hotchkiss v. Greenwood, 52 U.S. 248, 11 How. 248, 13 L. Ed. 683 (1851)*, and its progeny. See *383 U.S., at 15-17, 86 S. Ct. 684, 15 L. Ed. 2d 545*. The analysis is objective:

> [HN2] [**LEdHR2] [2]"Under *§ 103*, the scope and content of the prior art are to be determined; differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the pertinent art resolved. Against this background the obviousness or nonobviousness of the subject matter is determined. Such secondary considerations [***18] as commercial success, long felt but unsolved needs, failure of others, etc., might be utilized to give light to the circumstances surrounding the origin of the subject matter sought to be patented." *Id., at 17-18, 86 S. Ct. 684, 15 L. Ed. 2d 545*.

While the sequence of these questions might be reordered in any particular case, the factors continue to define the inquiry that controls. If a court, or patent examiner, conducts this analysis and concludes the claimed subject matter was obvious, the claim is invalid under *§ 103*.

Seeking to resolve the question of obviousness with more uniformity and consistency, the Court of Appeals for the Federal Circuit has employed an approach referred to by the parties as the "teaching, suggestion, or motivation" test (TSM test), under which a patent claim is only proved obvious if "some motivation or suggestion to combine the prior art teachings" can be found in the prior art, the nature of the problem, or the knowledge of a person having ordinary skill in the art. See, *e.g., Al-Site Corp. v. VSI Int'l, Inc., 174 F.3d 1308, 1323-1324 (CA Fed. 1999)*. KSR challenges that [*1735] test, or at least its application in this case. See *119 Fed. Appx. 282, 286-290 (CA Fed. 2005)*. [***19] Because the Court of Appeals addressed the question of obviousness in a manner contrary to *§ 103* and our precedents, we granted certiorari, *547 U.S. , 126 S. Ct. 2965, 165 L. Ed. 2d 949 (2006)*. We now reverse.

I

A

In car engines without computer-controlled throttles, the accelerator pedal interacts with the throttle via cable or other mechanical link. The pedal arm acts as a lever rotating around a pivot point. In a cable-actuated throttle control the rotation caused by pushing down the pedal pulls a cable, which in turn pulls open valves in the carburetor or fuel injection unit. The wider the valves open, the more fuel and air are released, causing combustion to increase and the car to accelerate. When the driver takes his foot off the pedal, the opposite occurs as the cable is released and the valves slide closed.

In the 1990's it became more common to install computers in cars to control engine operation. Computer-controlled throttles open and close valves in response to electronic signals, not through force transferred from the pedal by a mechanical link. Constant, delicate adjustments of air and fuel mixture are possible. The computer's rapid processing of factors beyond the pedal's position improves [***20] [**716] fuel efficiency and engine performance.

For a computer-controlled throttle to respond to a driver's operation of the car, the computer must know what is happening with the pedal. A cable or mechanical link does not suffice for this purpose; at some point, an electronic sensor is necessary to translate the mechanical operation into digital data the computer can understand.

Before discussing sensors further we turn to the mechanical design of the pedal itself. In the traditional design a pedal can be pushed down or released but cannot have its position in the footwell adjusted by sliding the pedal forward or back. As a result, a driver who wishes to be closer or farther from the pedal must either reposition himself in the driver's seat or move the seat in some way. In cars with deep footwells these are imperfect solutions for drivers of smaller stature. To solve the problem, inventors, beginning in the 1970's, designed pedals that could be adjusted to change their location in the footwell. Important for this case are two adjustable pedals disclosed in *U.S. Patent Nos. 5,010,782* (filed July 28, 1989) (Asano) and *5,460,061* (filed Sept. 17, 1993) (Redding). The Asano patent reveals a [***21] support structure that houses the pedal so that even when the pedal location is adjusted relative to the driver, one of the pedal's pivot points stays fixed. The pedal is also designed so that the force necessary to push the pedal down is the same regardless of adjustments to its location. The Redding patent reveals a different, sliding mechanism where both the pedal and the pivot point are adjusted.

We return to sensors. Well before Engelgau applied for his challenged patent, some inventors had obtained patents involving electronic pedal sensors for computer-controlled throttles. These inventions, such as the device disclosed in *U.S. Patent No. 5,241,936* (filed Sept. 9, 1991) ('936), taught that it was preferable to detect the pedal's position in the pedal assembly, not in the engine. The *'936 patent* disclosed a pedal with an electronic sensor on a pivot point in the pedal assembly. *U.S. Patent No. 5,063,811* (filed July 9, 1990) (Smith) taught that to prevent the [*1736] wires connecting the sensor to the computer from chafing and wearing out, and to avoid grime and damage from the driver's foot, the sensor should be put on a fixed part of the pedal assembly rather than in or on the pedal's [***22] footpad.

In addition to patents for pedals with integrated sensors inventors obtained patents for self-contained modular sensors. A modular sensor is designed independently of a given pedal so that it can be taken off the shelf and attached to mechanical pedals of various sorts, enabling the pedals to be used in automobiles with computer-controlled throttles. One such sensor was disclosed in *U.S. Patent No. 5,385,068* (filed Dec. 18, 1992) ('068). In 1994, Chevrolet manufactured a line of trucks using modular sensors "attached to the pedal support bracket, adjacent to the pedal and engaged with the pivot shaft about which the pedal rotates in operation." *298 F. Supp. 2d 581, 589 (ED Mich. 2003)*.

The prior art contained patents involving the placement of sensors on adjustable pedals as well. For example, *U.S. Patent No. 5,819,593* (filed Aug. 17, 1995) (Rixon) discloses an adjustable pedal assembly with an

[**717] electronic sensor for detecting the pedal's position. In the Rixon pedal the sensor is located in the pedal footpad. The Rixon pedal was known to suffer from wire chafing when the pedal was depressed and released.

This short account of pedal and sensor technology leads [***23] to the instant case.

B

KSR, a Canadian company, manufactures and supplies auto parts, including pedal systems. Ford Motor Company hired KSR in 1998 to supply an adjustable pedal system for various lines of automobiles with cable-actuated throttle controls. KSR developed an adjustable mechanical pedal for Ford and obtained *U.S. Patent No. 6,151,976* (filed July 16, 1999) ('976) for the design. In 2000, KSR was chosen by General Motors Corporation (GMC or GM) to supply adjustable pedal systems for Chevrolet and GMC light trucks that used engines with computer-controlled throttles. To make the '976 pedal compatible with the trucks, KSR merely took that design and added a modular sensor.

Teleflex is a rival to KSR in the design and manufacture of adjustable pedals. As noted, it is the exclusive licensee of the Engelgau patent. Engelgau filed the patent application on August 22, 2000 as a continuation of a previous application for *U.S. Patent No. 6,109,241*, which was filed on January 26, 1999. He has sworn he invented the patent's subject matter on February 14, 1998. The Engelgau patent discloses an adjustable electronic pedal described in the specification as a "simplified vehicle control [***24] pedal assembly that is less expensive, and which uses fewer parts and is easier to package within the vehicle." Engelgau, col. 2, lines 2-5, Supplemental App. 6. Claim 4 of the patent, at issue here, describes:

> "A vehicle control pedal apparatus comprising:
>
> a support adapted to be mounted to a vehicle structure;
>
> an adjustable pedal assembly having a pedal arm moveable in for[e] and aft directions with respect to said support;
>
> a pivot for pivotally supporting said adjustable pedal assembly with respect to said support and defining a pivot axis; and
>
> an electronic control attached to said support for controlling a vehicle system;
>
> said apparatus characterized by said electronic control being responsive to said pivot for providing a signal that corresponds to pedal arm position as said pedal arm pivots about said pivot [*1737] axis between rest and applied positions wherein the position of said pivot remains constant while said pedal arm moves in fore and aft directions with respect to said pivot." *Id.*, col. 6, lines 17-36, Supplemental App. 8 (diagram numbers omitted).

We agree with the District Court that the claim discloses "a position-adjustable pedal [***25] assembly with an electronic pedal position sensor attached to the support member of the pedal assembly. Attaching the sensor to the support member allows the sensor to remain in a fixed position while the driver adjusts the pedal." *298 F. Supp. 2d, at 586-587.*

Before issuing the Engelgau patent the U. S. Patent and Trademark Office (PTO) rejected one of the patent claims that was similar to, but [**718] broader than, the present claim 4. The claim did not include the requirement that the sensor be placed on a fixed pivot point. The PTO concluded the claim was an obvious combination of the prior art disclosed in Redding and Smith, explaining:

> "'Since the prior ar[t] references are from the field of endeavor, the purpose disclosed . . . would have been recognized in the pertinent art of Redding. Therefore it would have been obvious . . . to provide the device of Redding with the . . . means attached to a support member as taught by Smith.'" *Id., at 595.*

In other words Redding provided an example of an adjustable pedal and Smith explained how to mount a sensor on a pedal's support structure, and the rejected patent claim merely put these two teachings together.

[***26] Although the broader claim was rejected, claim 4 was later allowed because it included the limitation of a fixed pivot point, which distinguished the design from Redding's. *Ibid.* Engelgau had not included Asano among the prior art references, and Asano was not mentioned in the patent's prosecution. Thus, the PTO did not have before it an adjustable pedal with a fixed pivot point. The patent issued on May 29, 2001 and was assigned to Teleflex.

Upon learning of KSR's design for GM, Teleflex sent a warning letter informing KSR that its proposal would violate the Engelgau patent. "Teleflex believes that any supplier of a product that combines an adjustable pedal with an electronic throttle control necessarily

employs technology covered by one or more'" of Teleflex's patents. *Id., at 585*. KSR refused to enter a royalty arrangement with Teleflex; so Teleflex sued for infringement, asserting KSR's pedal infringed the Engelgau patent and two other patents. *Ibid.* Teleflex later abandoned its claims regarding the other patents and dedicated the patents to the public. The remaining contention was that KSR's pedal system for GM infringed claim 4 of the Engelgau patent. [***27] Teleflex has not argued that the other three claims of the patent are infringed by KSR's pedal, nor has Teleflex argued that the mechanical adjustable pedal designed by KSR for Ford infringed any of its patents.

C

The District Court granted summary judgment in KSR's favor. After reviewing the pertinent history of pedal design, the scope of the Engelgau patent, and the relevant prior art, the court considered the validity of the contested claim. [HN3] [**LEdHR3] [3] By direction of *35 U.S.C. § 282*, an issued patent is presumed valid. The District Court applied *Graham*'s framework to determine whether under summary-judgment standards KSR had overcome the presumption and demonstrated that claim 4 was obvious in light of the prior art in existence when [*1738] the claimed subject matter was invented. See *§ 102(a)*.

The District Court determined, in light of the expert testimony and the parties' stipulations, that the level of ordinary skill in pedal design was "'an undergraduate degree in mechanical engineering (or an equivalent amount of industry experience) [and] familiarity with pedal control systems for vehicles.'" *298 F. Supp. 2d, at 590*. The court then set forth the [***28] relevant prior art, including the patents and pedal designs described above.

[**719] Following *Graham*'s direction, the court compared the teachings of the prior art to the claims of Engelgau. It found "little difference." *298 F. Supp. 2d, at 590*. Asano taught everything contained in claim 4 except the use of a sensor to detect the pedal's position and transmit it to the computer controlling the throttle. That additional aspect was revealed in sources such as the *'068 patent* and the sensors used by Chevrolet.

Under the controlling cases from the Court of Appeals for the Federal Circuit, however, the District Court was not permitted to stop there. The court was required also to apply the TSM test. The District Court held KSR had satisfied the test. It reasoned (1) the state of the industry would lead inevitably to combinations of electronic sensors and adjustable pedals, (2) Rixon provided the basis for these developments, and (3) Smith taught a solution to the wire chafing problems in Rixon, namely locating the sensor on the fixed structure of the pedal. This could lead to the combination of Asano, or a pedal like it, with a pedal position sensor.

The conclusion that the [***29] Engelgau design was obvious was supported, in the District Court's view, by the PTO's rejection of the broader version of claim 4. Had Engelgau included Asano in his patent application, it reasoned, the PTO would have found claim 4 to be an obvious combination of Asano and Smith, as it had found the broader version an obvious combination of Redding and Smith. As a final matter, the District Court held that the secondary factor of Teleflex's commercial success with pedals based on Engelgau's design did not alter its conclusion. The District Court granted summary judgment for KSR.

With principal reliance on the TSM test, the Court of Appeals reversed. It ruled the District Court had not been strict enough in applying the test, having failed to make "'finding[s] as to the specific understanding or principle within the knowledge of a skilled artisan that would have motivated one with no knowledge of [the] invention' . . . to attach an electronic control to the support bracket of the Asano assembly." *119 Fed. Appx., at 288* (brackets in original) (quoting *In re Kotzab, 217 F.3d 1365, 1371 (CA Fed. 2000))*. The Court of Appeals held that the District Court was [***30] incorrect that the nature of the problem to be solved satisfied this requirement because unless the "prior art references address[ed] the precise problem that the patentee was trying to solve," the problem would not motivate an inventor to look at those references. *119 Fed. Appx., at 288*.

Here, the Court of Appeals found, the Asano pedal was designed to solve the "'constant ratio problem'"--that is, to ensure that the force required to depress the pedal is the same no matter how the pedal is adjusted--whereas Engelgau sought to provide a simpler, smaller, cheaper adjustable electronic pedal. *Ibid.* As for Rixon, the court explained, that pedal suffered from the problem of wire chafing but was not designed to solve it. In the court's view Rixon did not teach anything helpful to Engelgau's purpose. Smith, in turn, did not relate to adjustable pedals and did not "necessarily go to the issue of motivation [*1739] to attach the electronic control on the support bracket of the pedal assembly." *Ibid.* When the patents were interpreted in this way, the Court of Appeals held, they would not have led a person of ordinary skill to put a sensor on the sort of pedal described in Asano.

[**720] [***31] That it might have been obvious to try the combination of Asano and a sensor was likewise irrelevant, in the court's view, because ""[o]bvious to try" has long been held not to constitute obviousness.'" *Id., at 289* (quoting *In re Deuel, 51 F.3d 1552, 1559 (CA Fed. 1995))*.

The Court of Appeals also faulted the District Court's consideration of the PTO's rejection of the broader version of claim 4. The District Court's role, the Court of Appeals explained, was not to speculate regarding what the PTO might have done had the Engelgau patent mentioned Asano. Rather, the court held, the District Court was obliged first to presume that the issued patent was valid and then to render its own independent judgment of obviousness based on a review of the prior art. The fact that the PTO had rejected the broader version of claim 4, the Court of Appeals said, had no place in that analysis.

The Court of Appeals further held that genuine issues of material fact precluded summary judgment. Teleflex had proffered statements from one expert that claim 4 "'was a simple, elegant, and novel combination of features,'" *119 Fed. Appx., at 290*, compared to Rixon, [***32] and from another expert that claim 4 was nonobvious because, unlike in Rixon, the sensor was mounted on the support bracket rather than the pedal itself. This evidence, the court concluded, sufficed to require a trial.

II

A

We begin by rejecting the rigid approach of the Court of Appeals. Throughout this Court's engagement with the question of obviousness, our cases have set forth an expansive and flexible approach inconsistent with the way the Court of Appeals applied its TSM test here. To be sure, *Graham* recognized the need for "uniformity and definiteness." *383 U.S., at 18, 86 S. Ct. 684, 15 L. Ed. 2d 545*. Yet the principles laid down in *Graham* reaffirmed the "functional approach" of *Hotchkiss, 52 U.S. 248, 11 How. 248, 13 L. Ed. 683*. See *383 U.S., at 12, 86 S. Ct. 684, 15 L. Ed. 2d 545*. To this end, *Graham* set forth a broad inquiry and invited courts, where appropriate, to look at any secondary considerations that would prove instructive. *Id., at 17, 86 S. Ct. 684, 15 L. Ed. 2d 545*.

Neither the enactment of *§ 103* nor the analysis in *Graham* disturbed this Court's earlier instructions concerning the need for caution in granting a patent based on the combination of elements found in the prior art. For over a half century, [***33] the Court has held that [HN4] [**LEdHR4] [4] a "patent for a combination which only unites old elements with no change in their respective functions . . . obviously withdraws what is already known into the field of its monopoly and diminishes the resources available to skillful men." *Great Atlantic & Pacific Tea Co. v. Supermarket Equipment Corp., 340 U.S. 147, 152, 71 S. Ct. 127, 95 L. Ed. 162, 1951 Dec. Comm'r Pat. 572 (1950)*. This is a principal reason for declining to allow patents for what is obvious. The combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results. Three cases decided after *Graham* illustrate the application of this doctrine.

In *United States v. Adams, 383 U.S. 39, 40, 86 S. Ct. 708, 15 L. Ed. 2d 572, 174 Ct. Cl. 1293 (1966)*, a companion case to *Graham*, the Court considered the obviousness of a "wet battery" that varied from [**721] prior designs in two ways: [*1740] It contained water, rather than the acids conventionally employed in storage batteries; and its electrodes were magnesium and cuprous chloride, rather than zinc and silver chloride. The Court recognized that when a patent claims a structure already known in the prior art that is altered by the mere substitution of one [***34] element for another known in the field, the combination must do more than yield a predictable result. *383 U.S., at 50-51, 86 S. Ct. 708, 15 L. Ed. 2d 572, 174 Ct. Cl. 1293*It nevertheless rejected the Government's claim that Adams's battery was obvious. The Court relied upon the corollary principle that when the prior art teaches away from combining certain known elements, discovery of a successful means of combining them is more likely to be nonobvious. *Id., at 51-52, 86 S. Ct. 708, 15 L. Ed. 2d 572, 174 Ct. Cl. 1293*When Adams designed his battery, the prior art warned that risks were involved in using the types of electrodes he employed. The fact that the elements worked together in an unexpected and fruitful manner supported the conclusion that Adams's design was not obvious to those skilled in the art.

In *Anderson's-Black Rock, Inc. v. Pavement Salvage Co., 396 U.S. 57, 90 S. Ct. 305, 24 L. Ed. 2d 258 (1969)*, the Court elaborated on this approach. The subject matter of the patent before the Court was a device combining two pre-existing elements: a radiant-heat burner and a paving machine. The device, the Court concluded, did not create some new synergy: The radiant-heat burner functioned just as a burner was expected to function; and the paving machine did [***35] the same. The two in combination did no more than they would in separate, sequential operation. *Id., at 60-62, 90 S. Ct. 305, 24 L. Ed. 2d 258*. In those circumstances, "while the combination of old elements performed a useful function, it added nothing to the nature and quality of the radiant-heat burner already patented," and the patent failed under *§ 103*. *Id., at 62, 90 S. Ct. 305, 24 L. Ed. 2d 258* (footnote omitted).

Finally, in *Sakraida v. AG Pro, Inc., 425 U.S. 273, 96 S. Ct. 1532, 47 L. Ed. 2d 784 (1976)*, the Court derived from the precedents the conclusion that when a patent "simply arranges old elements with each performing the same function it had been known to perform" and yields no more than one would expect from such an arrangement, the combination is obvious. *Id., at 282, 96 S. Ct. 1532, 47 L .Ed. 2d 784*.

The principles underlying these cases are instructive when the question is whether a patent claiming the combination of elements of prior art is obvious. [HN5] [**LEdHR5] [5] When a work is available in one field of endeavor, design incentives and other market forces can prompt variations of it, either in the same field or a different one. If a person of ordinary skill can implement a predictable variation, *§ 103* likely bars its patentability. For [***36] the same reason, if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond his or her skill. *Sakraida* and *Anderson's-Black Rock* are illustrative--a court must ask whether the improvement is more than the predictable use of prior art elements according to their established functions.

Following these principles may be [**722] more difficult in other cases than it is here because the claimed subject matter may involve more than the simple substitution of one known element for another or the mere application of a known technique to a piece of prior art ready for the improvement. Often, it will be necessary for a court to look to interrelated teachings of multiple patents; the effects of demands known to the design community or present in the marketplace; and the background knowledge possessed by a person having [*1741] ordinary skill in the art, all in order to determine whether there was an apparent reason to combine the known elements in the fashion claimed by the patent at issue. To facilitate review, this analysis [***37] should be made explicit. See *In re Kahn, 441 F.3d 977, 988 (CA Fed. 2006)* ([HN6] [**LEdHR6] [6] "[R]ejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness"). As our precedents make clear, however, the analysis need not seek out precise teachings directed to the specific subject matter of the challenged claim, for a court can take account of the inferences and creative steps that a person of ordinary skill in the art would employ.

B

When it first established the requirement of demonstrating a teaching, suggestion, or motivation to combine known elements in order to show that the combination is obvious, the Court of Customs and Patent Appeals captured a helpful insight. See *Application of Bergel, 292 F.2d 955, 956-957, 48 C.C.P.A. 1102, 1961 Dec. Comm'r Pat. 504 (1961)*. As is clear from cases such as *Adams*, [HN7] [**LEdHR7] [7] a patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art. Although common sense directs one to look with care at a patent application that claims as innovation [***38] the combination of two known devices according to their established functions, it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does. This is so because inventions in most, if not all, instances rely upon building blocks long since uncovered, and claimed discoveries almost of necessity will be combinations of what, in some sense, is already known.

Helpful insights, however, need not become rigid and mandatory formulas; and when it is so applied, the TSM test is incompatible with our precedents. [HN8] [**LEdHR8] [8] The obviousness analysis cannot be confined by a formalistic conception of the words teaching, suggestion, and motivation, or by overemphasis on the importance of published articles and the explicit content of issued patents. The diversity of inventive pursuits and of modern technology counsels against limiting the analysis in this way. In many fields it may be that there is little discussion of obvious techniques or combinations, and it often may be the case that market demand, rather than scientific literature, will drive design trends. Granting patent protection [***39] to advances that would occur in the ordinary course without real innovation retards progress and may, in the case of patents combining previously known elements, deprive prior inventions of their value or utility.

In the years since the Court of Customs and Patent Appeals set forth the [**723] essence of the TSM test, the Court of Appeals no doubt has applied the test in accord with these principles in many cases. There is no necessary inconsistency between the idea underlying the TSM test and the *Graham* analysis. But when a court transforms the general principle into a rigid rule that limits the obviousness inquiry, as the Court of Appeals did here, it errs.

C

The flaws in the analysis of the Court of Appeals relate for the most part to the court's narrow conception of the obviousness inquiry reflected in its application of the TSM test. [HN9] [**LEdHR9] [9] In determining whether the subject matter of a patent claim is obvious, neither the particular motivation nor the avowed purpose of the [*1742] patentee controls. What matters is the objective reach of the claim. If the claim extends to what is obvious, it is invalid under *§ 103*. One of the ways in which a patent's subject matter can be proved obvious is [***40] by noting that there existed at the time of invention a known problem for which there was an obvious solution encompassed by the patent's claims.

The first error of the Court of Appeals in this case was to foreclose this reasoning by holding that courts and

patent examiners should look only to the problem the patentee was trying to solve. *119 Fed. Appx., at 288*. The Court of Appeals failed to recognize that [HN10] [**LEdHR10] [10] the problem motivating the patentee may be only one of many addressed by the patent's subject matter. The question is not whether the combination was obvious to the patentee but whether the combination was obvious to a person with ordinary skill in the art. Under the correct analysis, any need or problem known in the field of endeavor at the time of invention and addressed by the patent can provide a reason for combining the elements in the manner claimed.

The second error of the Court of Appeals lay in its assumption that a person of ordinary skill attempting to solve a problem will be led only to those elements of prior art designed to solve the same problem. *Ibid.* The primary purpose of Asano was solving the constant ratio problem; so, the court concluded, [***41] an inventor considering how to put a sensor on an adjustable pedal would have no reason to consider putting it on the Asano pedal. *Ibid.* Common sense teaches, however, that familiar items may have obvious uses beyond their primary purposes, and in many cases a person of ordinary skill will be able to fit the teachings of multiple patents together like pieces of a puzzle. Regardless of Asano's primary purpose, the design provided an obvious example of an adjustable pedal with a fixed pivot point; and the prior art was replete with patents indicating that a fixed pivot point was an ideal mount for a sensor. The idea that a designer hoping to make an adjustable electronic pedal would ignore Asano because Asano was designed to solve the constant ratio problem makes little sense. A person of ordinary skill is also a person of ordinary creativity, not an automaton.

The same constricted analysis led the Court of Appeals to conclude, in error, that a patent claim cannot be proved obvious merely by showing that the combination of elements was "obvious to try." *Id., at 289* (internal quotation marks omitted). [HN11] [**LEdHR11] [11] When there is a design need or market pressure to solve a problem [***42] and there are a finite number of identified, predictable [**724] solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to the anticipated success, it is likely the product not of innovation but of ordinary skill and common sense. In that instance the fact that a combination was obvious to try might show that it was obvious under *§ 103*.

The Court of Appeals, finally, drew the wrong conclusion from the risk of courts and patent examiners falling prey to hindsight bias. [HN12] [**LEdHR12] [12] A factfinder should be aware, of course, of the distortion caused by hindsight bias and must be cautious of arguments reliant upon *ex post* reasoning. See *Graham, 383 U.S., at 36, 86 S. Ct. 684, 15 L. Ed. 2d 545* (warning against a "temptation to read into the prior art the teachings of the invention in issue" and instructing courts to "'guard against slipping into the use of hindsight'" (quoting *Monroe Auto Equipment Co. v. Heckethorn Mfg. & Supply Co., 332 F.2d 406, 412 (CA6 1964))*). Rigid preventative rules that deny factfinders recourse to common sense, however, are [*1743] neither necessary under our case law nor consistent with it.

We note the [***43] Court of Appeals has since elaborated a broader conception of the TSM test than was applied in the instant matter. See, *e.g.*, *DyStar Textilfarben GmbH & Co. Deutschland KG v. C. H. Patrick Co., 464 F.3d 1356, 1367 (2006)* ("Our suggestion test is in actuality quite flexible and not only permits, but *requires*, consideration of common knowledge and common sense"); *Alza Corp. v. Mylan Labs., Inc., 464 F.3d 1286, 1291 (2006)* ("There is flexibility in our obviousness jurisprudence because a motivation may be found *implicitly* in the prior art. We do not have a rigid test that requires an actual teaching to combine . . ."). Those decisions, of course, are not now before us and do not correct the errors of law made by the Court of Appeals in this case. The extent to which they may describe an analysis more consistent with our earlier precedents and our decision here is a matter for the Court of Appeals to consider in its future cases. What we hold is that the fundamental misunderstandings identified above led the Court of Appeals in this case to apply a test inconsistent with our patent law decisions.

III

When we apply the standards we have [***44] explained to the instant facts, claim 4 must be found obvious. We agree with and adopt the District Court's recitation of the relevant prior art and its determination of the level of ordinary skill in the field. As did the District Court, we see little difference between the teachings of Asano and Smith and the adjustable electronic pedal disclosed in claim 4 of the Engelgau patent. A person having ordinary skill in the art could have combined Asano with a pedal position sensor in a fashion encompassed by claim 4, and would have seen the benefits of doing so.

A

Teleflex argues in passing that the Asano pedal cannot be combined with a sensor in the manner described by claim 4 because of the design of Asano's pivot mechanisms. See Brief for Respondents 48-49, and n 17. Therefore, Teleflex reasons, even if adding a sensor to Asano was obvious, that does not establish that claim 4 encompasses obvious subject matter. This argument was not, however, [**725] raised before the District Court. There Teleflex was content to assert only that the problem motivating the invention claimed by the Engelgau

patent would not lead to the solution of combining of Asano with a sensor. See Teleflex's Response [***45] to KSR's Motion for Summary Judgment of Invalidity in No. 02-74586 (ED Mich.), pp 18-20, App. 144a-146a. It is also unclear whether the current argument was raised before the Court of Appeals, where Teleflex advanced the nonspecific, conclusory contention that combining Asano with a sensor would not satisfy the limitations of claim 4. See Brief for Plaintiffs-Appellants in No. 04-1152 (CA Fed.), pp 42-44. Teleflex's own expert declarations, moreover, do not support the point Teleflex now raises. See Declaration of Clark J. Radcliffe, Ph.D., Supplemental App. 204-207; Declaration of Timothy L. Andresen, *id.*, at 208-210. The only statement in either declaration that might bear on the argument is found in the Radcliffe declaration:

> "Asano . . . and Rixon . . . are complex mechanical linkage-based devices that are expensive to produce and assemble and difficult to package. It is exactly these difficulties with prior art designs that [Engelgau] resolves. The use of an adjustable pedal with a single pivot reflecting pedal position combined with an electronic control mounted between the [*1744] support and the adjustment assembly at that pivot was a simple, elegant, and novel combination [***46] of features in the Engelgau*'565 patent*." *Id.*, at 206, P 16.

Read in the context of the declaration as a whole this is best interpreted to mean that Asano could not be used to solve "[t]he problem addressed by Engelgau'565[:] to provide a less expensive, more quickly assembled, and smaller package adjustable pedal assembly with electronic control." *Id.*, at 205, P 10.

The District Court found that combining Asano with a pivot-mounted pedal position sensor fell within the scope of claim 4. *298 F. Supp. 2d, at 592-593.* Given the sigificance of that finding to the District Court's judgment, it is apparent that Teleflex would have made clearer challenges to it if it intended to preserve this claim. In light of Teleflex's failure to raise the argument in a clear fashion, and the silence of the Court of Appeals on the issue, we take the District Court's conclusion on the point to be correct.

B

The District Court was correct to conclude that, as of the time Engelgau designed the subject matter in claim 4, it was obvious to a person of ordinary skill to combine Asano with a pivot-mounted pedal position sensor. There then existed a marketplace that created a strong [***47] incentive to convert mechanical pedals to electronic pedals, and the prior art taught a number of methods for achieving this advance. The Court of Appeals considered the issue too narrowly by, in effect, asking whether a pedal designer writing on a blank slate would have chosen both Asano and a modular sensor similar to the ones used in the Chevrolet truckline and disclosed in the *'068 patent*. The District Court employed this narrow inquiry as well, though it reached the correct result nevertheless. The proper question to have asked was whether a pedal designer of ordinary skill, facing the wide range of needs created by developments in the field of endeavor, [**726] would have seen a benefit to upgrading Asano with a sensor.

In automotive design, as in many other fields, the interaction of multiple components means that changing one component often requires the others to be modified as well. Technological developments made it clear that engines using computer-controlled throttles would become standard. As a result, designers might have decided to design new pedals from scratch; but they also would have had reason to make pre-existing pedals work with the new engines. Indeed, upgrading its [***48] own pre-existing model led KSR to design the pedal now accused of infringing the Engelgau patent.

For a designer starting with Asano, the question was where to attach the sensor. The consequent legal question, then, is whether a pedal designer of ordinary skill starting with Asano would have found it obvious to put the sensor on a fixed pivot point. The prior art discussed above leads us to the conclusion that attaching the sensor where both KSR and Engelgau put it would have been obvious to a person of ordinary skill.

The *'936 patent* taught the utility of putting the sensor on the pedal device, not in the engine. Smith, in turn, explained to put the sensor not on the pedal's footpad but instead on its support structure. And from the known wire-chafing problems of Rixon, and Smith's teaching that "the pedal assemblies must not precipitate any motion in the connecting wires," Smith, col. 1, lines 35-37, Supplemental App. 274, the designer would know to place the sensor on a nonmoving part of the pedal structure. The most obvious nonmoving point on the structure from which a sensor can [*1745] easily detect the pedal's position is a pivot point. The designer, accordingly, would follow Smith [***49] in mounting the sensor on a pivot, thereby designing an adjustable electronic pedal covered by claim 4.

Just as it was possible to begin with the objective to upgrade Asano to work with a computer-controlled throttle, so too was it possible to take an adjustable electronic

pedal like Rixon and seek an improvement that would avoid the wire-chafing problem. Following similar steps to those just explained, a designer would learn from Smith to avoid sensor movement and would come, thereby, to Asano because Asano disclosed an adjustable pedal with a fixed pivot.

Teleflex indirectly argues that the prior art taught away from attaching a sensor to Asano because Asano in its view is bulky, complex, and expensive. The only evidence Teleflex marshals in support of this argument, however, is the Radcliffe declaration, which merely indicates that Asano would not have solved Engelgau's goal of making a small, simple, and inexpensive pedal. What the declaration does not indicate is that Asano was somehow so flawed that there was no reason to upgrade it, or pedals like it, to be compatible with modern engines. Indeed, Teleflex's own declarations refute this conclusion. Dr. Radcliffe states that [***50] Rixon suffered from the same bulk and complexity as did Asano. See *id.*, at 206. Teleflex's other expert, however, explained that Rixon was itself designed by adding a sensor to a pre-existing mechanical pedal. See *id.*, at 209. If Rixon's base pedal was not too flawed to upgrade, then Dr. Radcliffe's declaration does not show Asano was either. Teleflex may have made a plausible argument that Asano is inefficient as compared [**727] to Engelgau's preferred embodiment, but to judge Asano against Engelgau would be to engage in the very hindsight bias Teleflex rightly urges must be avoided. Accordingly, Teleflex has not shown anything in the prior art that taught away from the use of Asano.

Like the District Court, finally, we conclude Teleflex has shown no secondary factors to dislodge the determination that claim 4 is obvious. Proper application of *Graham* and our other precedents to these facts therefore leads to the conclusion that claim 4 encompassed obvious subject matter. As a result, the claim fails to meet the requirement of *§ 103*.

We need not reach the question whether the failure to disclose Asano during the prosecution of Engelgau voids the presumption of validity given [***51] to issued patents, for claim 4 is obvious despite the presumption. We nevertheless think it appropriate to note that the rationale underlying the presumption--that the PTO, in its expertise, has approved the claim--seems much diminished here.

IV

A separate ground the Court of Appeals gave for reversing the order for summary judgment was the existence of a dispute over an issue of material fact. We disagree with the Court of Appeals on this point as well. To the extent the court understood the *Graham* approach to exclude the possibility of summary judgment when an expert provides a conclusory affidavit addressing the question of obviousness, it misunderstood the role expert testimony plays in the analysis. [HN13] [**LEdHR13] [13] In considering summary judgment on that question the district court can and should take into account expert testimony, which may resolve or keep open certain questions of fact. That is not the end of the issue, however. The ultimate judgment of obviousness is a legal determination. *Graham, 383 U.S., at 17, 86 S. Ct. 684, 15 L. Ed. 2d 545*. Where, as here, the content of the prior art, the scope of the patent [*1746] claim, and the level of ordinary skill in the art are not in material dispute, and [***52] the obviousness of the claim is apparent in light of these factors, summary judgment is appropriate. Nothing in the declarations proffered by Teleflex prevented the District Court from reaching the careful conclusions underlying its order for summary judgment in this case.

* * *

We build and create by bringing to the tangible and palpable reality around us new works based on instinct, simple logic, ordinary inferences, extraordinary ideas, and sometimes even genius. These advances, once part of our shared knowledge, define a new threshold from which innovation starts once more. And [HN14] [**LEdHR14] [14] as progress beginning from higher levels of achievement is expected in the normal course, the results of ordinary innovation are not the subject of exclusive rights under the patent laws. Were it otherwise patents might stifle, rather than promote, the progress of useful arts. See *U.S. Const., Art. I, § 8, cl. 8*. These premises led to the bar on patents claiming obvious subject matter established in *Hotchkiss* and codified in *§ 103*. Application of the bar must not be confined within a test or formulation too constrained to serve its purpose.

KSR provided convincing evidence that mounting a modular [***53] sensor on a fixed pivot point of the Asano pedal was a design step well within the [**728] grasp of a person of ordinary skill in the relevant art. Its arguments, and the record, demonstrate that claim 4 of the Engelgau patent is obvious. In rejecting the District Court's rulings, the Court of Appeals analyzed the issue in a narrow, rigid manner inconsistent with *§ 103* and our precedents. The judgment of the Court of Appeals is reversed, and the case remanded for further proceedings consistent with this opinion.

It is so ordered.

REFERENCES

35 U.S.C.S. § 103

Chisum on Patents §§ 5.02-5.04, 11.06 (Matthew Bender)

L Ed Digest, Patents § 19.1

L Ed Index, Patents

Supreme Court's views as to what is patentable subject matter under federal law as "process," "machine," "manufacture," or "composition of matter." *65 L. Ed. 2d 1197.*

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DYSTAR TEXTILFARBEN GMBH & CO DEUTSCHLAND KG, Plaintiff-Appellee, v. C.H. PATRICK CO., and BANN QUIMICA LTDA, Defendants-Appellants.

06-1088

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

464 F.3d 1356; 2006 U.S. App. LEXIS 24642; 80 U.S.P.Q.2D (BNA) 1641

October 3, 2006, Decided

SUBSEQUENT HISTORY: Rehearing denied by, Rehearing, en banc, denied by *Dystar Textilfarben GmbH v. C.H. Patrick Co., 2006 U.S. App. LEXIS 32267 (Fed. Cir., Dec. 4, 2006)*
US Supreme Court certiorari denied by *Dystar Textilfarben GmbH & Co. Deutschland KG v. C.H. Patrick Co., 127 S. Ct. 2937, 168 L. Ed. 2d 262, 2007 U.S. LEXIS 7532 (U.S., June 11, 2007)*

PRIOR HISTORY: [**1] Appealed from: United States District Court for the District of South Carolina. Magistrate Judge William M. Catoe, Jr.
DyStar Textilfarben GmbH & Co. Deutschland KG v. C. H. Patrick Co., 2005 U.S. Dist. LEXIS 43662 (D.S.C., Nov. 1, 2005)

DISPOSITION: REVERSED.

CASE SUMMARY:

PROCEDURAL POSTURE: Plaintiff patent holder sued defendant manufacturer, alleging patent infringement. The United States District Court for the District of South Carolina granted plaintiff judgment as a matter of law that it had not engaged in inequitable conduct before the United States Patent and Trademark Office. A jury found that defendant had infringed the patent and declined to hold the patent claims invalid for, inter alia, obviousness. Defendant appealed.

OVERVIEW: Defendant argued that four claims were obvious. The appellate court concluded that the jury's implicit finding that the level of ordinary skill in the art was a dyer was unsupported by substantial evidence because an ordinary artisan was a person designing an optimal textile dyeing process with some expertise in chemistry. The jury's corresponding decision to disregard the primary cited prior art as nonanalogous was also erroneous. Under the suggestion test, the prior art did not teach away from the combination of two patented processes. Under the correct level of ordinary skill, it would have been obvious from the earlier patented processes, in view of other references, to stabilize catalytically hydrogenated leuco indigo solution through vacuum conditions and to introduce the solution directly into the dyebath. The presence of certain secondary considerations of nonobviousness were insufficient to overcome the conclusion that the evidence only supported a legal conclusion that process claim 1 would have been obvious. Claims 2-4 were also invalid for obviousness as they did not recite a nonobvious invention beyond that cited in claim 1.

OUTCOME: The denial of defendant's motion for judgment as a matter of law of invalidity of claims 1-4 for obviousness was reversed.

LexisNexis(R) Headnotes

Patent Law > Jurisdiction & Review > Standards of Review > General Overview
[HN1] The United States Court of Appeals for the Federal Circuit reviews decisions on motions for judgment as a matter of law and motions for a new trial under the law of the regional circuit.

Patent Law > Jurisdiction & Review > Standards of Review > De Novo Review
[HN2] In the Fourth Circuit, the grant or denial of a judgment as a matter of law (JMOL) in a patent infringement action is reviewed de novo, which requires the appellate court to step into the shoes of the trial judge

and reapply the JMOL standard. The question is whether a jury, viewing the evidence in the light most favorable to the plaintiff, could have properly reached the conclusion reached by the jury. The appellate court must reverse if a reasonable jury could only rule in favor of the defendant; if reasonable minds could differ, the appellate court must affirm.

Patent Law > Jurisdiction & Review > Standards of Review > Abuse of Discretion
[HN3] In the context of a patent infringement action, the denial of a motion for a new trial is reviewed in the Fourth Circuit for abuse of discretion.

Patent Law > Jurisdiction & Review > Standards of Review > De Novo Review
Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
[HN4] A determination that a claimed invention would have been obvious, and thus the patent issued thereon invalid, is a legal conclusion that the United States Court of Appeals for the Federal Circuit reviews de novo. The Federal Circuit must determine if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. *35 U.S.C.S. § 103(a)*. The Federal Circuit thus considers whether a person of ordinary skill in the art would have been motivated to combine the prior art to achieve the claimed invention and whether there would have been a reasonable expectation of success in doing so.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN5] Obviousness depends on (1) the scope and content of the prior art; (2) the differences between the claimed invention and the prior art; (3) the level of ordinary skill in the art; and (4) any relevant secondary considerations, including commercial success, long felt but unsolved needs, and failure of others.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN6] The United States Court of Appeals for the Federal Circuit articulates a subsidiary requirement for the first Graham factor, the scope and content of the prior art. Where all claim limitations are found in a number of prior art references, the factfinder must determine what the prior art teaches, whether it teaches away from the claimed invention, and whether it motivates a combination of teachings from different references. It is important in this inquiry to distinguish between the references sought to be combined and the prior art, as the latter category is much broader. For example, textbooks or treatises may include basic principles unlikely to be restated in cited references.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN7] As the United States Court of Appeals for the Federal Circuit explains, the suggestion test, as the motivation-to-combine inquiry has come to be known, prevents statutorily proscribed hindsight reasoning when determining the obviousness of an invention. This test informs the Graham analysis by implementing the recognition of the importance of guarding against hindsight, as is evident in its discussion of the role of secondary considerations as serving to guard against slipping into use of hindsight and to resist the temptation to read into the prior art the teachings of the invention in issue.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN8] In contrast to the characterization of some commentators, the suggestion test is not a rigid categorical rule. The motivation need not be found in the references sought to be combined, but may be found in any number of sources, including common knowledge, the prior art as a whole, or the nature of the problem itself. There is no requirement that the prior art contain an express suggestion to combine known elements to achieve the claimed invention. Rather, the suggestion to combine may come from the prior art, as filtered through the knowledge of one skilled in the art.

Civil Procedure > Appeals > Standards of Review > General Overview
[HN9] When the jury does not make explicit factual findings in the form of answers to written interrogatories or special verdicts, the appellate court must discern the jury's implied factual findings by interpreting the evidence consistently with the verdict and drawing all reasonable inferences in the prevailing party's favor.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN10] Where claim limitations are found in a combination of prior art references, the factfinder must determine what the prior art teaches, whether it teaches away from

the claimed invention, and whether it motivates a combination of teachings from different references.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN11] In the context of determining whether a patent is obvious, evidence of a motivation to combine need not be found in the prior art references themselves, but rather may be found in the knowledge of one of ordinary skill in the art, or, in some cases, from the nature of the problem to be solved. When not from the prior art references, the evidence of motive will likely consist of an explanation of the well-known principle or problem-solving strategy to be applied.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN12] The United States Court of Appeals for the Federal Circuit's suggestion test is in actuality quite flexible and not only permits, but requires, consideration of common knowledge and common sense in determining a patent's obviousness.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN13] The United States Court of Appeals for the Federal Circuit repeatedly holds that an implicit motivation to combine exists not only when a suggestion may be gleaned from the prior art as a whole, but when the improvement is technology-independent and the combination of references results in a product or process that is more desirable, for example because it is stronger, cheaper, cleaner, faster, lighter, smaller, more durable, or more efficient. Because the desire to enhance commercial opportunities by improving a product or process is universal, and even common-sensical, the Federal Circuit holds that there exists in these situations a motivation to combine prior art references even absent any hint of suggestion in the references themselves. In such situations, the proper question is whether the ordinary artisan possesses knowledge and skills rendering him capable of combining the prior art references.

Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN14] Although the United States Court of Appeals for the Federal Circuit customarily discusses a motivation to combine as part of the first Graham factor in determining obviousness, the scope and content of the prior art, motivation to combine is also inextricably linked to the level of ordinary skill. If, as is usually the case, no prior art reference contains an express suggestion to combine references, then the level of ordinary skill will often predetermine whether an implicit suggestion exists. Persons of varying degrees of skill not only possess varying bases of knowledge, they also possess varying levels of imagination and ingenuity in the relevant field, particularly with respect to problem-solving abilities.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN15] The United States Court of Appeals for the Federal Circuit must evaluate obviousness on a claim-by-claim basis.

COUNSEL: William T. Enos, Oblon, Spivak, McClelland, Maier & Neustadt, P.C., of Alexandria, Virginia, argued for plaintiff-appellee. With him on the brief were Richard D. Kelly and Andrew K. Beverina.

Neil C. Jones, Nelson Mullins Riley & Scarborough, L.L.P., of Greenville, South Carolina, argued for defendants-appellants.

JUDGES: Before MICHEL, Chief Judge, RADER and SCHALL, Circuit Judges. Opinion for the court filed by Chief Judge MICHEL. Concurring opinion filed by Circuit Judge SCHALL.

OPINION BY: MICHEL

OPINION

[*1357] MICHEL, Chief Judge.

DyStar Textilfarben GmbH & Co. Deutschland KG ("DyStar") sued defendants C.H. Patrick Co. and Bann Quimica Ltda. (collectively, "Bann") in the United States District Court for the District of South Carolina, alleging direct, contributory, and induced infringement of *U.S. Patent No. 5,586,992* ("the *'992 patent*"), [1] which discloses a process for dyeing textile materials with catalytically hydrogenated leuco indigo. DyStar and Bann Quimica Ltda. are large chemical manufacturers [*1358] that, inter alia, sell prereduced indigo for use in dyeing [**2] processes; C.H. Patrick Co. purchased prereduced indigo solution from Bann Quimica Ltda. in 2002 and used it to dye yarn in a process alleged to infringe.

1 BASF was the assignee of the *'992 patent*. BASF divested its dyestuff business, including the *'992 patent*, to DyStar in 2000.

The parties agreed to a jury trial before a magistrate judge. Prior to charging the jury and in open court, the

magistrate judge granted DyStar's motion for judgment as a matter of law ("JMOL") that it had not engaged in inequitable conduct before the United States Patent and Trademark Office ("PTO"). The jury rendered a verdict that "Bann Quimica and/or C.H. Patrick" had infringed each of claims 1-4, assessed damages at $ 90,000, and declined to hold the *'992 patent* claims invalid for lack of enablement, [**3] anticipation or obviousness. DyStar Textilfarben GmbH & Co Deutschland KG v. C.H. Patrick Co., Civ. No. 6:02-2946-WMC (D.S.C. Sept. 16, 2005).

Following briefing, the magistrate judge denied Bann's motions for JMOL or, alternatively, a new trial on the question of invalidity of the *'992 patent* for anticipation, obviousness, and lack of enablement. *Dystar Textilfarben Gmbh v. C. H. Patrick Co., Civ. No. 6:02-2946-WMC, 2005 U.S. Dist. LEXIS 43662 (D.S.C. Nov. 1, 2005).* The magistrate judge did not issue an opinion. His order stated:

> The jury diligently considered the evidence presented and found for the plaintiff. This court concludes that the jury's verdict was reasonable and was supported by evidence in the record. Therefore, as this court has great respect for trial by jury and the right of the parties to request a jury trial, this court will not substitute its findings for those of the jury as the jury's decision was clearly supported by the trial record and was reasonable.

Id.

Bann appeals from the denials of its motions on anticipation and obviousness, and the grant of JMOL to DyStar regarding inequitable conduct. For the reasons explained below, we reverse the district court's denial of Bann's motion for JMOL of invalidity of claims 1-4 for obviousness.

I

Indigo has been used in dyeing textile materials for thousands of years. Because indigo pigment is insoluble in water, it must be de-oxidized, or "reduced," to a water-soluble white form known as "leuco indigo" before it can be used in dyeing. Leuco indigo is unstable; it oxidizes [**4] and returns to its blue pigment form when exposed to oxygen. Thus, leuco indigo solution needs to be kept in an oxygen-free environment, or otherwise stabilized, if it is not being used immediately for dyeing.

For many years, dyehouses commonly reduced indigo in-house through a process known as hydrosulfite reduction. Dyers created a "stock vat," in which indigo is reduced in water with sodium hydrosulfite and solubilized with an alkali, e.g., sodium hydroxide. The resulting leuco indigo solution is then transferred into a feed tank and fed into the dyebath. After the dyebath is prepared, the textile material is dyed through a process known as "dipping" and "skying." In "dipping," the textile material is contacted with leuco indigo in the dyebath; in "skying," the dyed textile material is introduced to the air, causing the indigo to convert back to its blue pigment form.

A second common method of indigo reduction, catalytic hydrogenation, was patented by Andre Brochet in 1917. See *U.S. Patent No. 1,247,927* ("Brochet"). The superficial difference between hydrosulfite reduction and catalytic hydrogenation is that the latter uses gaseous hydrogen, [*1359] rather than sodium hydrosulfite, [**5] as a reducing agent. Catalytic hydrogenation allowed "economical production of concentrated solutions of leuco derivatives free from impurities and mineral salts"; when left to settle, the solution naturally separates from nickel or another catalytic metal and can be "drawn off and is ready for use". Brochet, ll.88-90, 109-110. Most important to the dyehouses, however, was the fact that Brochet's leuco indigo solution could be stabilized in solid form, usually powder or paste, and coated with molasses or glue to protect the reduced indigo from air and prevent premature oxidation. This allowed the indigo reduction process to shift out of the dyehouses and into chemical manufacturers, which began to produce and sell prereduced indigo to dyehouses in the early 1900s. Rather than create a stock vat, dyers needed only to dissolve the prereduced indigo into a preparation tank, add caustic soda (i.e., sodium hydroxide) and sodium hydrosulfite to remove oxygen from the water, and transfer the resulting solution from the preparation tank to the dyebath. This significantly reduced the time necessary to prepare a dyebath, the dyehouses' expenditures on sodium hydrosulfite and caustic soda, and [**6] the level of pollution in dyehouse waste water and on dyehouse floors.

The process of dyeing textile materials with catalytically hydrogenated leuco indigo traditionally has involved six steps: (1) reducing indigo to its leuco form in solution; (2) stabilizing the leuco indigo solution, usually in paste or powder form; (3) creating a preparation tank in which the dried leuco indigo is re-converted to solution form; (4) adding the solution to the dyebath; (5) dipping; and (6) skying.

Claim 1 of the *'992 patent*, the only independent claim at issue, recites:

> A process for dyeing cellulose-containing textile material with indigo which comprises
>
> a) introducing into a dyebath an aqueous solution of leuco indigo solution prepared by catalytic hydrogenation;
>
> b) contacting the textile material with the dyebath; and, after the leuco indigo has gone onto the textile material,
>
> c) converting said leuco indigo back into the pigment form in a conventional manner by air oxidation.

'992 patent, col.6, l.66-col.7, l.6. The *'992 patent* thus improved upon the prior art by eliminating steps two and three of the traditional process: stabilizing the leuco indigo solution [**7] into a paste or powder form, and then reconstituting the solution in a preparation tank. Instead, it allowed a dyer to pour prereduced indigo solution directly into a dyebath and commence dyeing immediately.

II

Bann appeals from the denials of its motions on anticipation of claim 1 and obviousness of claims 1-4, and the grant of DyStar's JMOL of no inequitable conduct. [HN1] We review decisions on motions for JMOL and motions for a new trial under the law of the regional circuit. *MicroStrategy, Inc. v. Bus. Objects, S.A., 429 F.3d 1344, 1348 (Fed. Cir. 2005) (JMOL); EMI Group N. Am., Inc. v. Cypress Semiconductor Corp., 268 F.3d 1342, 1348 (Fed. Cir. 2001)* (new trial). [HN2] In the Fourth Circuit, the grant or denial of JMOL is reviewed de novo, which requires us to step into the shoes of the trial judge and reapply the JMOL standard. *Johnson v. MBNA Am. Bank, NA, 357 F.3d 426, 431 (4th Cir. 2004).* "The question is whether a jury, viewing the evidence in the light most favorable to [DyStar], could have properly reached the conclusion reached by this [*1360] jury. We must reverse if a reasonable jury could only rule in favor of [Bann]; if reasonable [**8] minds could differ, we must affirm." Id. (internal citation and quotation marks omitted). [HN3] The denial of a motion for a new trial is reviewed in the Fourth Circuit for abuse of discretion. *United States v. Perry, 335 F.3d 316, 320 (4th Cir. 2003).*

Bann asserts that claim 1 of the *'992 patent* is invalid because it is anticipated by Brochet. Bann further argues that claims 1-4 are invalid as obvious in light of Brochet and certain other prior art, including two pre-1917 BASF patents--United States Patent Nos. 820,900 ("Wimmer") and 885,978 ("Chaumat"), a post-World War II report of the British Intelligence Objectives Sub-Committee ("BIOS report"), and the 1936 Manual for the Dyeing of Cotton and Other Vegtable Fibres ("Manual"), published by General Dyestuff Corporation. We address the broader argument, relating to obviousness, first.

A

[HN4] A determination that a claimed invention would have been obvious, and thus the patent issued thereon invalid, is a legal conclusion that we review de novo. *Richardson-Vicks, Inc. v. The Upjohn Co., 122 F.3d 1476, 1479 (Fed. Cir. 1997).* We must determine "if the differences between the subject matter [**9] sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains." *35 U.S.C. § 103(a).* We thus consider whether a person of ordinary skill in the art would have been motivated to combine the prior art to achieve the claimed invention and whether there would have been a reasonable expectation of success in doing so. *Brown & Williamson Tobacco Corp. v. Philip Morris, Inc., 229 F.3d 1120, 1124 (Fed. Cir. 2000).*

Underpinning this legal inquiry are four groups of factual findings, which, in a jury trial, we review for substantial evidence. *Richardson-Vicks, 122 F.3d at 1479.* Following the 1952 enactment of *§ 103*, the Supreme Court explained that [HN5] obviousness depends on (1) the scope and content of the prior art; (2) the differences between the claimed invention and the prior art; (3) the level of ordinary skill in the art; and (4) any relevant secondary considerations, including commercial success, long felt but unsolved needs, and failure of others. *Graham v. John Deere Co., 383 U.S. 1, 17, 86 S. Ct. 684, 15 L. Ed. 2d 545 (1966).* [**10]

[HN6] This court has articulated a subsidiary requirement for the first Graham factor, the scope and content of the prior art. *SIBIA Neurosciences, Inc. v. Cadus Pharm. Corp., 225 F.3d 1349, 1356 (Fed. Cir. 2000).* Where, as here, all claim limitations are found in a number of prior art references, the factfinder must determine"[w]hat the prior art teaches, whether it teaches away from the claimed invention, and whether it motivates a combination of teachings from different references". *In re Fulton, 391 F.3d 1195, 1199-1200 (Fed. Cir. 2004).* It is important in this inquiry to distinguish between the references sought to be combined and "the

prior art", as the latter category is much broader. For example, textbooks or treatises may include basic principles unlikely to be restated in cited references.

[HN7] As we recently explained in *Alza Corp. v. Mylan Labs., Inc., No. 06-1019, 464 F.3d 1286, 2006 U.S. App. LEXIS 22616 (Fed. Cir. Sept. 6, 2006)*, the suggestion test-as our motivation-to-combine inquiry has come to be known-"prevent[s] statutorily proscribed hindsight reasoning when determining the obviousness of an [*1361] invention." *464 F.3d 1286, [WL] at *7.* This [**11] test "informs the Graham analysis", *464 F.3d 1286, [WL] at *8* (quoting *In re Kahn, 441 F.3d 977, 987 (Fed. Cir. 2006))*, by implementing the Supreme Court's recognition of "the importance of guarding against hindsight, as is evident in its discussion of the role of secondary considerations as 'serv[ing] to guard against slipping into use of hindsight and to resist the temptation to read into the prior art the teachings of the invention in issue.'" *464 F.3d 1286, [WL] at *6* (quoting *Graham, 383 U.S. at 36*).

[HN8] In contrast to the characterization of some commentators, the suggestion test is not a rigid categorical rule. The motivation need not be found in the references sought to be combined, but may be found in any number of sources, including common knowledge, the prior art as a whole, or the nature of the problem itself. *In re Dembiczak, 175 F.3d 994, 999 (Fed. Cir. 1999).* As we explained in *Motorola, Inc. v. Interdigital Tech. Corp., 121 F.3d 1461, 1472 (Fed. Cir. 1997)*, "there is no requirement that the prior art contain an express suggestion to combine known elements to achieve the claimed invention. Rather, the suggestion to combine [**12] may come from the prior art, as filtered through the knowledge of one skilled in the art."

For one to conclude that the invention of the *'992 patent* would have been obvious, then, the prior art, common knowledge, or the nature of the problem, viewed through the eyes of an ordinary artisan, must have suggested the following steps: (1) creating leuco indigo solution through catalytic hydrogenation; (2) stabilizing the leuco indigo in solution form; (3) adding the leuco indigo solution directly into a dyebath; (4) dipping; and (5) skying. [HN9] Because the jury did not make explicit factual findings in the form of answers to written interrogatories or special verdicts, we must discern the jury's implied factual findings by interpreting the evidence consistently with the verdict and drawing all reasonable inferences in DyStar's favor. *Konkel v. Bob Evans Farms Inc., 165 F.3d 275, 279 (4th Cir. 1999).*

B

Bann's obviousness argument rests primarily on three U.S. patents: Brochet, Wimmer, and Chaumat. Brochet is directed to "the Manufacture of Leuco Derivatives of Vat Dyestuffs" generally, of which indigo is one. Brochet, ll.6-7. By its plain language, Brochet discloses [**13] the process of preparing an aqueous solution of leuco indigo by catalytic hydrogenation. There can be no serious dispute that the ultimate use of a "dyestuff" is dyeing textile materials; indeed, the *'992 patent* inventor, Georg Schnitzer, testified that leuco indigo solutions were known to be used in 1917 for dyeing, and Dr. Richard Blackburn, one of DyStar's technical experts, testified that BASF began reducing indigo with catalytic hydrogenation, and selling the reduced indigo to dyehouses, in 1926. Moreover, both parties agree that dipping and skying were well known in the art. As explained supra, when indigo is reduced in-house in a stock vat, the resulting leuco indigo solution is introduced directly into the dyebath without first being stabilized through drying. Thus, the critical issue in our obviousness analysis is whether stabilizing catalytically reduced leuco indigo in solution form, rather than in powder form coated with molasses, for example, renders the claimed process nonobvious to one of ordinary skill in the art.

1. Level of Ordinary Skill in the Art

Because the parties disagree over the relevance of the cited prior art, which, [*1362] fundamentally, is a disagreement [**14] over the level of ordinary skill in the art, we address this third *Graham* factor first. DyStar asserts that we must disregard Brochet, Wimmer, and Chaumat, because a person of ordinary skill in the art would not be aware of these references. In short, DyStar argues that no knowledge of chemistry is required in the relevant technical field.

DyStar points to testimony from Dr. Blackburn that "[a] person of ordinary skill in the art is a dyer", someone with "a high school degree" who is "able to read and write", but whose knowledge is limited to "flipping the switches" on the machine. Dr. Blackburn also testified, however, that a person of ordinary skill in the art was "running the dye processes", which required, inter alia, "doing the calculations". When confronted with the inconsistency between his testimony regarding the applicable level of skill during cross-examination, Dr. Blackburn stated that "it's difficult to say" which of the two skill levels should be applied to the obviousness inquiry. Dr. Blackburn explained that "those people may do both jobs", but concluded by stating, "I think the former [i.e., the person 'flipping the switches']" is the ordinary artisan. Thus, [**15] the jury had evidence before it of two potential levels of ordinary skill: (1) that of a dyer and (2) that of a person creating the dye processes, who we will refer to as a dyeing process designer.

DyStar presented evidence that The Application of Vat Dyes, a book by the American Association of Textile Chemists and Colorists, teaches people how to dye and is

a text that a dyer would have had at the time of the *'992 patent* invention. Dr. Nolan Etters, Bann's expert witness, agreed that "a person of ordinary skill in the art" would be a member of the American Association of Textile Chemists and Colorists and conceded that none of the prior art cited by Bann is referenced in The Application of Vat Dyes.

We agree with DyStar that we are required to assume the jury accepted its argument that a person of ordinary skill in the art is a dyer with no knowledge of chemistry. Because the jury heard testimony that the prior art cited by Bann was directed toward chemists, not dyers, the jury must have found the prior art cited by Bann neither in the relevant art nor analogous arts and then, consistent with the limited evidence of prior art deemed relevant, concluded that the process claimed [**16] in the *'992 patent* would not have been obvious.

However, substantial evidence does not support the jury's finding that a person of ordinary skill is a dyer with no knowledge of chemistry. Indeed, that factual finding is inconsistent with the *'992 patent*'s very purpose. The technical problem that the process of the *'992 patent* and the prior art cited by Bann sought to solve is precisely the same: an improved process for dyeing textile materials with indigo. This process includes several discrete subcomponents--e.g., indigo reduction and dyebath preparation--and an ordinary artisan would be concerned with all of them. To beneficially practice the dyeing process claimed in the *'992 patent*, the ordinary artisan must have a higher-level perspective, as he must first decide whether it is more efficient to reduce indigo in-house or purchase prereduced indigo and, if prereduced, must then decide whether solid or solution form is preferable.

Designing an optimal dyeing process requires knowledge of chemistry and systems engineering, for example, and by no means can be undertaken by a person of only high school education whose skill set is limited to "flipping the switches". This is especially [**17] true when one considers that only in the last century have improvements [*1363] in indigo reduction chemistry enabled outsourcing of the indigo reduction step from dyehouses to chemical manufacturers; prior to that simplification, there would have been no question that a dyer would also require knowledge of indigo reduction. Because, for this patent, the only finding supported by substantial evidence is that an ordinary artisan is not a dyer but a person designing an optimal dyeing process, the jury's implicit finding of a mere dyer cannot withstand scrutiny on JMOL. Accordingly, the jury's apparent decision to disregard Brochet, Wimmer, and Chaumat, and perhaps other prior art references, as neither in the dye process art nor even in analogous arts is unsupported by substantial evidence.

2. Scope and Content of Prior Art

[HN10] Where, as here, claim limitations are found in a combination of prior art references, the factfinder must determine "[w]hat the prior art teaches, whether it teaches away from the claimed invention, and whether it motivates a combination of teachings from different references". *Fulton, 391 F.3d at 1199-1200 (Fed. Cir. 2004).*

To support its argument [**18] that an ordinary artisan--i.e., a dyeing process designer--would have known to attempt to stabilize the Brochet solution in oxygen-excluding conditions for addition directly into a dyebath, Bann points to two pre-Brochet BASF patents. Wimmer, issued in 1906, discloses a leuco indigo solution that "can be filtered and the filtrate (which contains a high percentage of indigo white) can be placed on the market without any further treatment", in contrast to reduction using zinc, which required the solution "to be separated before the solution can be used for dyeing." Wimmer, ll.34-40. Chaumat, issued in 1908, discloses a leuco indigo solution that "may be drawn off protected from the air and preserved indefinitely in receptacles which are either soldered or closed in any other hermetic manner." Chaumat, ll.84-87. Although Wimmer and Chaumat disclose different reducing methods than Brochet-Wimmer suggests the use of iron, rather than zinc, as a reducing agent, and Chaumat discloses an electrolytic process for indigo reduction--Bann argues that once the indigo has been converted to its leuco form, the distinction is irrelevant for dyeing purposes. Accordingly, Bann argues that this prior [**19] art would teach an ordinary artisan in the field of indigo dyeing process design to attempt to stabilize any leuco indigo solution, however reduced, for addition directly into the dyebath.

a. What does the prior art teach?

DyStar argues that because Wimmer and Chaumat involve different methods of reducing indigo, they are nonanalogous art and properly disregarded by the jury. In support of this assertion, Mr. Schnitzer testified that, up until the time of the invention, BASF's "people from production" believed that leuco indigo created from catalytic hydrogenation was too unstable to be added directly to the dyebath, and might "stain the yarn with indigo pigment" or "block[] pipes". DyStar offered evidence that, prior to the *'992 patent*, BASF had limited its sales of catalytically hydrogenated leuco indigo to that stabilized in paste or solid form. Thus, argues DyStar, the language in Wimmer and Chaumat suggesting that the solution could be stabilized and sold in solution form does not apply to catalytically hydrogenated leuco indigo solution.

We disagree. As explained supra, the proper focus is on the indigo dyeing process as a whole, which requires the [**20] ordinary artisan to consider (and choose between) the various indigo reduction methods. The *'992 patent* is directed toward [*1364] a process of dyeing with indigo and, although a specific method of reduction is required by claim 1, the first step in the process requires indigo in prereduced form. It is undisputed that reduced indigo by any reduction method, not just catalytic hydrogenation, has been used in the indigo dyeing process. The prior art involving indigo reduction by other methods is thus not merely analogous art, it is the same art. Accordingly, all limitations recited by claim 1 of the *'992 patent*--including the immediate use of leuco indigo solution for dyeing--are contained in the prior art. [2]

> 2 Because the only difference between the claimed invention and the cited prior art is the method of indigo reduction, which we have held is irrelevant to an indigo dyeing process employing prereduced indigo, we do not separately discuss this second Graham factor.

b. Does the prior art teach away from the claimed invention?

We reject DyStar's assertion that contemporaneous articles by Wimmer and Brochet teach away from the combination [**21] of Brochet and Chaumat, and thus the claimed process. DyStar acknowledges that no specific language in these references teaches away from the invention of the *'992 patent*. Rather, because these references do not discuss the stabilization of leuco indigo solution (in solution form) for immediate addition to a dyebath, DyStar somehow concludes that these references teach that leuco indigo solution "cannot be used to dye but is instead useful only as an intermediate."

Although Wimmer's contemporaneous article only describes the use of indigo solution as an intermediate product, he does not retract his patent language indicating that "the solution can be filtered and the filtrate (which contains a high percentage of indigo white) can be placed on the market without any further treatment". Wimmer, ll.33-37. Likewise, the Brochet patent, directed toward all vat dyestuffs, broadly teaches that the process "produce[s] mother-liquors which can be diluted immediately before use, or be treated by evaporation under reduced pressure or by any other means to obtain concentrated products for sale." Brochet, ll.66-70. This language implies that all vat dyestuffs, including indigo, may either be [**22] used immediately for dyeing or concentrated prior to sale. In his contemporaneous article, Brochet stated that his catalytically hydrogenated solution could be used "economically to obtain concentrated indigo white [i.e., leuco indigo] solutions that are free of impurities and alkaline salts, that can be concentrated in vacuum in order to obtain white indigo as a paste". This mere failure to discuss immediate use of his leuco indigo solution for dyeing is not the same thing as Brochet stating in his article that, though most dyestuffs may be used immediately or stored in oxygen-excluding containers, his leuco indigo solution may only be concentrated in paste form. We will not read into a reference a teaching away from a process where no such language exists.

c. Is there a motivation to combine?

DyStar argues that this court's "suggestion test" for obviousness requires the cited references themselves to contain a suggestion, teaching, or motivation to combine them, and that it must be explicitly stated. DyStar then points out, correctly, that Brochet does not suggest combining his invention with those of Chaumat or Wimmer to stabilize his leuco indigo solution [**23] in oxygen-excluding containers until either using it directly in the dyebath or placing it on the market for sale, respectively. [*1365] Absent such a teaching, urges DyStar, the invention of claim 1 of the *'992 patent* cannot be obvious.

DyStar's argument misreads this court's cases and misdescribes our suggestion test, echoing notions put forth recently by various commentators and accepted in major reports. A 2003 report by the Federal Trade Commission, for example, quoted testimony of certain witnesses that this court requires "specific and definitive [prior] art references with clear motivation of how to combine those references" and requires the PTO to find "the glue expressly leading you all the way [to obviousness]" and "connect the dots . . . very, very clearly." Fed. Trade Comm'n, To Promote Innovation: The Proper Balance of Competition and Patent Law and Policy ch. 4, at 11 (2003). Similarly, a 2004 report by the National Academy of Sciences summarized views of a few commentators that "standards of patentability--especially the non-obviousness standard--have become too lax as a result of court decisions" by the Federal Circuit, leading to the deterioration of patent quality. [**24] Nat'l Research Council, A Patent System for the 21st Century 3 (Stephen A. Merrill et al. eds., 2004). But see Am. Intellectual Prop. Law Ass'n, AIPLA Response to the National Academies Report entitled "A Patent System for the 21st Century" 10 (2004) ("AIPLA believes that the courts, including the Federal Circuit, have applied the standard of non-obviousness with both the needed rigor and the appropriate vigor, and they have done so with a commendable consistency over the past two decades. If a difficulty exists with application of the non-obviousness standard today, it does not lie in the patent statute or in substantive law of non-obviousness as applied in the courts.")

Seeking to support their assertions about Federal Circuit caselaw, these few commentators have quoted isolated statements from three of our precedents in particular, including *Dembiczak, 175 F.3d at 1000*, wherein we stated that the analysis by the Board of Patent Appeals and Interferences ("Board") "fails to demonstrate how the [two cited] references teach or suggest their combination" (emphasis added), and *In re Lee, 277 F.3d 1338, 1341, 1344 (Fed. Cir. 2002),* [**25] where we characterized the Board's statement that "[t]he conclusion of obviousness may be made from common knowledge and common sense of a person of ordinary skill in the art without any specific hint or suggestion in a particular reference" as "[o]mission of a relevant factor required by precedent". They have also cited our repeated use of the word "references" in the following list from *Ruiz v. A.B. Chance Co., 234 F.3d 654 (Fed. Cir. 2000),* where we suggested a motivation to combine may be found:

> 1) in the prior art references themselves;
>
> 2) in the knowledge of those of ordinary skill in the art that certain references, or disclosures in those references, are of special interest or importance in the field; or
>
> 3) from the nature of the problem to be solved, leading inventors to look to references relating to possible solutions to that problem.

Id. at 665 (emphasis added) (internal quotation marks omitted).

Despite containing arguably imprecise language in these statements, quoted out of context, each of the above-cited cases correctly applies the suggestion test and by no means requires an explicit teaching [**26] to combine to be found in a particular prior art reference. Dembiczak involved the combination of a reference in the plastic trash bag art with children's arts and crafts books that included, among innumerable fanciful drawings, jack-o-lanterns. [*1366] Contrary to some interpretations, we stated explicitly that [HN11] evidence of a motivation to combine need not be found in the prior art references themselves, but rather may be found in "the knowledge of one of ordinary skill in the art, or, in some cases, from the nature of the problem to be solved." *175 F.3d at 999* (citation omitted). When not from the prior art references, the "evidence" of motive will likely consist of an explanation of the well-known principle or problem-solving strategy to be applied. Our analysis in Dembiczak focused on an explicit teaching in the prior art not because our case law requires it, but because the Board had stated that "the [two cited] references would have suggested the application of . . . facial indicia to the prior art plastic trash bags." *Id. at 1000* (emphasis added). We held the Board's obviousness determination legally insufficient because, in addition [**27] to failing to make Graham findings, the Board's analysis was "limited to a discussion of the ways that the multiple prior art references can be combined to read on the claimed invention", "rather than pointing to specific information in [the two references] that suggest the combination". Id. On appeal to this court, the Commissioner of Patents and Trademarks attempted to defend the Board decision by laying out, using the Graham factors, a clear--and likely affirmable--rationale establishing the level of ordinary skill and explaining the motivation to combine. *Id. at 1001*. We declined to consider these arguments, newly raised on appeal, stating that they did "little more than highlight the shortcomings of the decision below." Id.

In Ruiz, as in Dembiczak, we vacated a conclusion of obviousness because the factfinder failed to make Graham factor findings. *234 F.3d at 660*. Far from requiring evidence of an explicit motivation to combine, we suggested in Ruiz that there may have existed an implicit motivation to combine, based on testimony that the invention was an improvement over the prior art because it is "easy to [**28] install" and "low cost". *Id. at 666*. We explained that such "[e]vidence which suggests that the combination of two references would suggest the resulting improvement is one way in which to determine a reason, suggestion, or motivation to combine" and instructed the district court to consider the evidence on remand. Id.

Likewise, a close reading of In re Lee reveals that our objection was not to the Board's statement that "[t]he conclusion of obviousness may be made from common knowledge and common sense of a person of ordinary skill in the art without any specific hint or suggestion in a particular reference", but its utter failure to explain the "common knowledge and common sense" on which it relied. See *277 F.3d at 1341, 1344*. Lee involved a patent combining a prior art video game instruction handbook describing a "demonstration mode" with a prior art television set having a menu display allowing video and audio adjustments. The Board, without comment, adopted the Examiner's Answer, which merely stated that the combination of the two cited references "'would have been obvious to one of ordinary skill in the art since the demonstration [**29] mode is just a programmable feature which can be used in many different devices for providing automatic introduction by adding the proper programming software' and that 'another motivation would be that the automatic demonstration mode is user friendly and it functions as a tutorial.'" *Id. at 1341*. We explained that "[c]onclusory statements such as those here provided do not fulfill the agency's obligation" to

explain all material facts relating to a motivation to combine. *Id. at 1344.* In other words, we instructed the Board to explain why "common sense" of an ordinary artisan [*1367] seeking to solve the problem at hand would have led him to combine the references. We noted that our predecessor court held more than thirty years earlier that "common knowledge and common sense" were sufficient to establish a motivation to combine, *In re Bozek, 57 C.C.P.A. 713, 416 F.2d 1385 (C.C.P.A. 1969)*, and distinguished that case because, in Bozek, the examiner first "established that this knowledge was in the art". *Id. at 1390.* We instructed that assumptions about common sense cannot substitute for evidence thereof, as the Board attempted [**30] to do in *Lee. 277 F.3d at 1345*; see also *In re Zurko, 258 F.3d 1379, 1383, 1385 (Fed. Cir. 2001)* (reversing as unsupported by substantial evidence a finding of motivation to combine cited references, where the Board adopted Examiner's unsupported assertion that claim limitation missing from cited references was "basic knowledge" and it "would have been nothing more than good common sense" to combine the references, and explaining that "[t]his assessment of basic knowledge and common sense was not based on any evidence in the record"); *In re Rouffet, 149 F.3d 1350, 1357 (Fed. Cir. 1998)* (affirming finding of high level of ordinary skill and the Board's explanation as to why cited reference implicitly would suggest missing claim limitation to ordinary artisan, but reversing its reliance on high level of ordinary skill as basis of motivation to combine, and stating, "The Board did not, however, explain what specific understanding or technological principle within the knowledge of one of ordinary skill in the art would have suggested the combination. Instead, the Board merely invoked the high level of skill in the field of art. If [**31] such a rote invocation could suffice to supply a motivation to combine, the more sophisticated scientific fields would rarely, if ever, experience a patentable technical advance.").

It is difficult to see how our suggestion test could be seen as rigid and categorical given the myriad cases over several decades in which panels of this court have applied the suggestion test flexibly. Obviousness is a complicated subject requiring sophisticated analysis, and no single case lays out all facets of the legal test. DyStar's argument and the above-cited commentary highlight the danger inherent in focusing on isolated dicta rather than gleaning the law of a particular area from careful reading of the full text of a group of related precedents for all they say that is dispositive and for what they hold. When parties like Dystar do not engage in such careful, candid, and complete legal analysis, much confusion about the law arises and, through time, can be compounded. [3]

3 Indeed, the United States Supreme Court recently granted certiorari in a case involving this court's application of the suggestion test. *KSR Int'l Co. v. Teleflex, Inc., 126 S. Ct. 2965, 165 L. Ed. 2d 949, 2006 U.S. LEXIS 4912 (June 26, 2006).* In KSR, we vacated a district court's grant of summary judgment of invalidity for obviousness. The district court found a motivation to combine not in the references but "largely on the nature of the problem to be solved", which we did not deem erroneous. *Teleflex, Inc. v. KSR Int'l Co., 119 Fed. App'x 282, 287 (Fed. Cir. 2005)* (unpublished). Rather, we vacated because the court did not explain sufficiently its rationale, and failed to make "findings as to the specific understanding or principle within the knowledge of a skilled artisan that would have motivated one with no knowledge of [the] invention to make the combination in the manner claimed." *Id. at 288* (citation omitted).

[**32] [HN12] Our suggestion test is in actuality quite flexible and not only permits, but requires, consideration of common knowledge and common sense. See, e.g., *In re Kotzab, 217 F.3d 1365, 1369 (Fed. Cir. 2000)* ("A critical step in analyzing the patentability of claims pursuant to *section 103(a)* is casting [*1368] the mind back to the time of invention, to consider the thinking of one of ordinary skill in the art, guided only by the prior art references and the then-accepted wisdom in the field."); *Motorola, 121 F.3d at 1472* ("[T]he suggestion to combine may come from the prior art, as filtered through the knowledge of one skilled in the art."); *Bozek, 416 F.2d at 1390* ("Having established that this knowledge was in the art, the examiner could then properly rely, as put forth by the solicitor, on a conclusion of obviousness 'from common knowledge and common sense of the person of ordinary skill in the art without any specific hint or suggestion in a particular reference.'").

Indeed, [HN13] we have repeatedly held that an implicit motivation to combine exists not only when a suggestion may be gleaned from the prior art as a whole, but when the [**33] "improvement" is technology-independent and the combination of references results in a product or process that is more desirable, for example because it is stronger, cheaper, cleaner, faster, lighter, smaller, more durable, or more efficient. Because the desire to enhance commercial opportunities by improving a product or process is universal--and even common-sensical--we have held that there exists in these situations a motivation to combine prior art references even absent any hint of suggestion in the references themselves. In such situations, the proper question is whether the ordinary artisan possesses knowledge and skills rendering him capable of combining the prior art references.

In *Pro-Mold & Tool Co., Inc. v. Great Lakes Plastics, Inc., 75 F.3d 1568 (Fed. Cir. 1996)*, for example,

we analyzed asserted obviousness of a claimed invention of a sports trading card holder only slightly larger than the trading card. We stated that "[w]e start from the self-evident proposition that mankind, in particular, inventors, strive to improve that which already exists". *Id. at 1573*. We required no documentary evidence of motive. We explained that the [**34] motivation to combine "a reference describing an elegant card holder and cover arrangement with a reference describing a card holder no larger than necessary to enclose the card . . . was thus evident from the very size of the card itself." [4] *Id. at 1573*. Because an ornamental card holder just large enough to enclose the card would be more efficient, there existed an implicit, indeed common-sensical, motivation to combine the two references.

4 We vacated the district court's grant of summary judgment of invalidity for other reasons, finding genuine disputes of material fact on the issue of commercial success. *Id. at 1573*.

Similarly, in *Sandt Tech., Ltd. v. Resco Metal & Plastics Corp., 264 F.3d 1344, 1355 (Fed. Cir. 2001)*, we held invalid for obviousness a patent claim for a stainless steel cover for pay telephones. The only relevant difference between the claimed invention and the prior art covers was that the former attached the steel cover to the telephone via studs and the latter attached it with welds. Id. We noted that the difference between attaching with welds and studs was merely a "slight variation [**35] that produced convenience". Id. We found a clear motive to alter the prior art welded cover simply because "[u]sing studs was a cheaper, faster, and more convenient method of attachment", id., absent even a hint of suggestion to combine.

In *Mazzari v. Rogan, 323 F.3d 1000 (Fed. Cir. 2003)*, we affirmed a district court grant of summary judgment of invalidity for obviousness of a patent claiming the use of underwater acoustic waves to kill zebra mussels. The Board had upheld the rejection of an application based on two prior art patents: an acoustic wave generator [*1369] that enabled altering wave intensity and focusing acoustic energy along a particular angle, and a method of using water-borne acoustic waves to kill zebra mussels. The Board held that a motivation to combine the two references existed because an ordinary artisan would have been aware of both references and that combination of the two was "more efficient". *Id. at 1002-03*. The inventor then brought a civil suit against the Director of the PTO pursuant to *35 U.S.C. § 145*, and the district court granted summary judgment in favor of the Director. We deemed sufficient [**36] the Board's explanation of a motivation to combine and affirmed "because the references illustrate that it is well known in the art to use acoustic energy to kill and repel zebra mussels." *Id. at 1006*; see also *Ruiz, 234 F.3d at 666* (remanding for determination of whether testimony that claimed invention was "easy to install" and "low cost" established motivation to combine).

In situations where a motivation to combine is based on these principles, the invention cannot be said to be nonobvious. Our precedent on this point, moreover, is consistent with the Supreme Court's holdings in Graham and three other obviousness decisions pre-dating the establishment of this court. See *Sakraida v. AG Pro, Inc., 425 U.S. 273, 282, 96 S. Ct. 1532, 47 L. Ed. 2d 784 (1976)*; *Anderson's-Black Rock, Inc. v. Pavement Salvage Co. 396 U.S. 57, 90 S. Ct. 305, 24 L. Ed. 2d 258 (1969)*; *United States v. Adams, 383 U.S. 39, 86 S. Ct. 708, 15 L. Ed. 2d 572, 174 Ct. Cl. 1293 (1966)*.

In Anderson's-Black Rock, the Supreme Court held invalid for obviousness a patent covering (1) a radiant burner for heating asphalt (2) attached to the front of a standard asphalt-paving machine. Both [**37] elements were well-known in the prior art individually, with the difference being that previously, radiant-heat burners were not used in paving, but merely for patching limited areas of asphalt. *396 U.S. at 58-59*. Because asphalt is usually laid sequentially in parallel strips, the adjoining strip cools before the next strip is laid, leading to what is known as a "cold joint"--an area of poor bonding into which water and dirt enter, causing deterioration. *Id. at 57-58*. By reheating the adjoining edge of the earlier-laid strip as a new strip is laid, the invention sought to eliminate the cold joint. No explicit suggestion to combine the prior art references would have been necessary because the invention merely improved the efficiency of the already-known process of laying pavement through the already-known method of merging two sections of asphalt through re-heating the earlier laid section--both of which would have been common knowledge to ordinary artisans in the field of laying asphalt. See also *Graham, 383 U.S. at 24-25* (holding invalid for obviousness Graham's patent disclosing a spring clamp on a plow shank, where claimed invention [**38] merely improved mechanical weakness in prior Graham patent, because ordinary artisan would have had mechanical skills sufficient to "immediately see that the thing to do was what Graham did"); *id. at 36-37* (holding invalid for obviousness Cook Chemical's patent disclosing a plastic finger sprayer with a "hold-down" lid serving as a built-in dispenser for bottles of liquid products, where differences from prior art were "exceedingly small and quite nontechnical" and device was "old in the art").

Likewise, in Sakraida, the Supreme Court held invalid for obviousness a patent for a barn having "a paved, sloped barn floor with downhill drains", "elevated" cow stalls, and a "dam" behind which water may be stored and abruptly released in order to "send a sheet of water

cascading through the dairy sweeping the manure to the downhill drains." *Ag Pro, Inc. v. Sakraida,* [*1370] *474 F.2d 167, 168 (5th Cir. 1973)* (quoting *U.S. Patent No. 3,223,070*), rev'd by *425 U.S. 273, 96 S. Ct. 1532, 47 L. Ed. 2d 784*. Because the cleaning action from an uphill release of water was superior to that from a hose, the claimed invention reduced the quantity of water necessary to clean the barn floor [**39] and obviated additional hand labor, e.g., brooms or shovels, shortening the cleaning process from a few hours to a few minutes. Id. The Supreme Court nonetheless negated patent protection, characterizing the invention as "the work of the skillful mechanic, not that of the inventor." *Sakraida, 425 U.S. at 279* (internal quotation marks omitted). The Court aptly noted that "[e]xploitation of the principle of gravity adds nothing to the sum of useful knowledge". Id. Under this court's case law, there would have been no need for "evidence" of a motivation to combine a prior art reference with a universally-known physical principle to achieve more powerful and simultaneous sweep of water.

In Adams, a companion case to Graham, the Supreme Court upheld the validity of a patent for a nonrechargeable water-activated battery having magnesium and cuprous chloride electrodes. *Adams, 383 U.S. at 51.* The Court recognized that "each of the elements of the Adams battery was well known in the prior art", but rejected the United States' obviousness argument because the prior art taught away from the Adams patent's combination. As the Court succinctly [**40] stated:

> To combine [the references] as did Adams required that a person reasonably skilled in the prior art must ignore that (1) batteries which continued to operate on an open circuit and which heated in normal use were not practical; and (2) water-activated batteries were successful only when combined with electrolytes detrimental to the use of magnesium.

Id. at 51-52. The Court instructed that such "known disadvantages in old devices . . . may be taken into account in determining obviousness", *id. at 52*, and we have incorporated this notion into our case law. See, e.g., *Fulton, 391 F.3d at 1199-1200* (instructing the factfinder to determine "[w]hat the prior art teaches, whether it teaches away from the claimed invention, and whether it motivates a combination of teachings from different references").

[HN14] Although this court customarily discusses a motivation to combine as part of the first Graham factor, the scope and content of the prior art, see *SIBIA Neurosciences, 225 F.3d at 1356*, motivation to combine is also inextricably linked to the level of ordinary skill. If, as is usually the [**41] case, no prior art reference contains an express suggestion to combine references, then the level of ordinary skill will often predetermine whether an implicit suggestion exists. Persons of varying degrees of skill not only possess varying bases of knowledge, they also possess varying levels of imagination and ingenuity in the relevant field, particularly with respect to problem-solving abilities. If the level of skill is low, for example that of a mere dyer, as DyStar has suggested, then it may be rational to assume that such an artisan would not think to combine references absent explicit direction in a prior art reference. If, however, as we have held as a matter of law, the level of skill is that of a dyeing process designer, then one can assume comfortably that such an artisan will draw ideas from chemistry and systems engineering--without being told to do so.

A dyeing process designer would have been aware that reducing leuco indigo in-house was time-consuming as well as expensive and that it created much pollution [*1371] on the dyehouse floor and in public sewers. He would have known that purchasing solid prereduced indigo would save time, space, and money: dyers would no longer spend [**42] time creating stock vats, and the dyehouse would require far less hydrosulfite and caustic soda. A dyeing process designer reading Chaumat would have learned that leuco indigo solution "may be drawn off protected from the air and preserved indefinitely in receptacles which are either soldered or closed in any other hermetic manner." Chaumat, ll.84-87. From his chemistry background, he would have known how to close off a receptacle hermetically. He would have known that, if he could thus stabilize leuco indigo solution, he would save even more time, space, and money: dyers would no longer need stock vats or preparation tanks because they could simply pour the prereduced solution directly into the dyebath itself, and they would no longer need to purchase any hydrosulfite or caustic soda. A dyeing process designer reading Brochet would have realized that catalytic hydrogenation provided advantages over other methods of indigo reduction in that the leuco indigo was "free from impurities and mineral salts". Brochet, l.90. Naturally, then, an ordinary artisan with knowledge of Chaumat, reading Brochet, would have realized that, by stabilizing catalytic hydrogenated leuco indigo solution [**43] in oxygen-excluding containers, he could devise a "cheaper, faster, and more convenient" indigo dyeing process. See *Sandt, 264 F.3d at 1355.* Although the *'992 patent* claimed a new, more efficient, way of performing a known function, dyeing indigo--the asserted innovation, storing leuco indigo solution in airtight containers for immediate use in dyebaths, is merely "exploitation" of the well-known princi-

ple of vacuum packaging. See *Sakraida, 425 U.S. at 279.* In sum, it is the work of a skilled chemist, not of an inventor.

3. Secondary Considerations of Nonobviousness

The presence of certain secondary considerations of nonobviousness are insufficient as a matter of law to overcome our conclusion that the evidence only supports a legal conclusion that claim 1 would have been obvious. To be sure, the jury heard testimony that DyStar has enjoyed considerable commercial success from the introduction of its product, and all parties agree that eighty years elapsed between Brochet's invention and another inventor's thought to vacuum-seal the Brochet solution and add it directly to the dyebath. However, Mr. Schnitzer's testimony that BASF's "people [**44] from production"--who we here assume arguendo were dyeing process designers--believed that leuco indigo solution added directly to the dyebath might "stain the yarn with indigo pigment" or "block[] pipes" was a bare assertion that is not only undocumented and non-specific, but also unsupported by even a brief explanation of the chemistry underlying this assumption. As such, it does not constitute substantial evidence of a secondary consideration favoring nonobviousness.

Moreover, another secondary consideration cited by DyStar--i.e., failed attempts--actually detracts from its argument, and heavily so. DyStar points out that another chemical company, Buffalo Color, abandoned a 1979 effort to market a prereduced indigo solution made by hydrosulfite reduction. The record shows, however, that Buffalo was only mildly concerned with instability problems--it noted only that the instability of leuco indigo "would require special (and costly) shipping conditions to protect it from oxidation". Rather, Buffalo decided against selling a leuco indigo solution because it would involve increased shipping costs, might require customers to invest in additional [*1372] storage facilities, and would cost [**45] more to produce, likely forcing it to increase prices to customers. Buffalo's decision was thus not a failed attempt, but a calculated business judgment to abandon a potential new product line.

C

Our inquiry does not end here, however, because [HN15] we must evaluate obviousness on a claim-by-claim basis. *Dayco Prods., Inc. v. Total Containment, Inc., 329 F.3d 1358, 1370 (Fed. Cir. 2003)* ("[D]ependent or multiple dependent claims shall be presumed valid even though dependent upon an invalid claim.").

Claims 2-4 depend from process claim 1. Claim 2 requires that the resulting solution contain from 10% to 35% by weight of leuco indigo; claim 3 requires that the solution contain from 2% to 10% by weight of alkali; and claim 4 requires that the solution contain from 2% to 10% by weight of sodium hydroxide. Dr. Blackburn, DyStar's own expert witness, confirmed that the plain language of Wimmer sets forth a "30 percent aqueous solution of leuco indigo", which falls within the range prescribed by claim 2. Dr. Blackburn also testified that the solution disclosed by Wimmer contains "5.1 percent" by weight of alkali--the claim language says "at least six and a half percent", [**46] both of which fall between 2% and 10%, as required by claim 3. Likewise, Wimmer indicates that his example solution uses "NaOH", sodium hydroxide, and Mr. Schnitzer agreed with Bann's counsel's assertion that the term "caustic" is "chemically the same thing as sodium hydroxide", which meets the requirement of claim 4. DyStar does not dispute these concessions on appeal. Thus, given DyStar's testimony and the plain language of Wimmer, claims 2-4 do not recite a nonobvious invention beyond that recited in claim 1. Accordingly, claims 2-4 must also be held invalid for obviousness as a matter of law.

III

In sum, because an ordinary artisan is a person designing an optimal textile dyeing process with some expertise in chemistry, the jury's implicit finding that the level of ordinary skill in the art is a dyer is unsupported by substantial evidence; its corresponding decision to disregard the primary cited prior art as nonanalogous was also erroneous. Under the correct level of ordinary skill, it would have been obvious from Chaumat and Brochet, in view of Wimmer and other references, to stabilize catalytically hydrogenated leuco indigo solution through vacuum conditions and to introduce [**47] the solution directly into the dyebath.

Because all claims are held invalid for obviousness, we need not address alleged anticipation of claim 1 or lack of enablement as to claims 1-4. Likewise, whether the *'992 patent* is unenforceable due to inequitable conduct need not be decided. Finally, we do not address the request for a new trial as all asserted claims are held invalid as a matter of law for obviousness. Accordingly, the trial court's denial of JMOL that claims 1-4 of the *'992 patent* are invalid for obviousness is reversed.

REVERSED.

CONCUR BY: SCHALL

CONCUR

SCHALL, Circuit Judge, concurring.

I concur in the judgment of reversal. See *Alza Corp. v. Mylan Labs., Inc., No. 06-1088, 2006 U.S. App. LEXIS 22616, at 4-7 (Fed. Cir. Sept. 6, 2006)*; *In re Kahn, 441*

F.3d 977, 987-88 (Fed. Cir. 2006); Cross Med. Prods., [*1373] *Inc. v. Medtronic Sofamor Danek, Inc., 424 F.3d 1293, 1322 (Fed. Cir. 2005).*

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U.S. Appln. No. 10/770,639
Reference No. 15

LEXSEE 225 F.3D 1349

SIBIA NEUROSCIENCES, INC., Plaintiff-Appellee, v. CADUS PHARMACEUTICAL CORPORATION, Defendant-Appellant.

99-1381

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

225 F.3d 1349; 2000 U.S. App. LEXIS 22516; 55 U.S.P.Q.2D (BNA) 1927

September 6, 2000, Decided

PRIOR HISTORY: [**1] Appealed from: United States District Court for the Southern District of California. Judge Irma E. Gonzales.

DISPOSITION: REVERSED.

CASE SUMMARY:

PROCEDURAL POSTURE: Defendant appealed the judgment of the United States District Court for the Southern District of California entered in plaintiff's favor after a jury found that the patent claims at issue concerning a cell-based screening method were infringed and were not invalid.

OVERVIEW: Plaintiff owned a patent which was directed to a cell-based screening method useful for the identification of compounds that exhibited agonist and antagonist activity with respect to particular cell surface proteins. According to the patent, the claimed methods were particularly effective because they allowed a scientist to rapidly and reliably screen large numbers of compounds for agonist and antagonist activity. Plaintiff sued defendant for patent infringement. Defendant argued that the claims of the patent were invalid as obvious under *35 U.S.C.S. § 103(a)* or as not enabled under *35 U.S.C.S. § 112*. A jury rejected defendant's invalidity defenses of obviousness and non-enablement. The court held that a patent claim was invalid if the differences between the subject matter sought to be patented and the prior art were such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art. The court reversed judgment, holding that the asserted claims were obvious as a matter of law because plaintiff based its patent on a prior art reference and there was evident motivation to modify the prior art reference.

OUTCOME: Judgment reversed; defendant did not infringe plaintiff's patent because the patent's claims were obvious as a matter of law since the patent was based on a prior art reference and there was evident motivation for plaintiff to modify the prior art reference.

LexisNexis(R) Headnotes

Civil Procedure > Trials > Judgment as Matter of Law > General Overview
Civil Procedure > Appeals > Standards of Review > De Novo Review
Civil Procedure > Appeals > Standards of Review > Substantial Evidence > General Overview
[HN1] An appellate court reviews the denial of a motion for judgment as a matter of law following a jury verdict by reapplying the district court's standard of review. Thus, an appellate court reviews issues of law de novo. With regard to factual findings, an appellate court must presume that the jury resolved all factual disputes in favor of the prevailing party, and the appellate court must leave those findings undisturbed as long as they are supported by substantial evidence.

Civil Procedure > Appeals > Standards of Review > Substantial Evidence > General Overview
[HN2] A factual finding is supported by substantial evidence if a reasonable jury could have found in favor of the prevailing party in light of the evidence presented at trial. Substantial evidence is more than a mere scintilla. It means such relevant evidence as a reasonable mind might accept as adequate to support a conclusion. Thus, substantial evidence review involves an examination of the record as a whole, taking into consideration evidence that both justifies and detracts from the decision of the fact-finder.

Civil Procedure > Trials > Judgment as Matter of Law > General Overview
Civil Procedure > Appeals > Standards of Review
[HN3] In reviewing the record on a denial of a motion for judgment as a matter of law, an appellate court must draw all reasonable inferences in favor of the prevailing party, and not make credibility determinations or substitute its view of the conflicting evidence for that of the jury. If, however, after reviewing all of the evidence in a light most favorable to the prevailing party, the court is convinced that a reasonable jury could not have found in that party's favor, the court must reverse the denial of judgment as a matter of law.

Civil Procedure > Appeals > Standards of Review > De Novo Review
Patent Law > Infringement Actions > Defenses > Patent Invalidity > Fact & Law Issues
Patent Law > Jurisdiction & Review > Standards of Review > De Novo Review
[HN4] In patent law, the first step in any invalidity analysis is claim construction, an issue of law that an appellate court reviews de novo.

Patent Law > Nonobviousness > Elements & Tests > Claimed Invention as a Whole
Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN5] A patent claim is invalid if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art. *35 U.S.C.S. § 103(a).*

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Nonobviousness > Elements & Tests > General Overview
Patent Law > Nonobviousness > Evidence & Procedure > Fact & Law Issues
[HN6] While the ultimate conclusion of obviousness is for the court to decide as a matter of law, several factual inquiries underlie this determination. These inquiries include the scope and content of the prior art, the level of ordinary skill in the field of the invention, the differences between the claimed invention and the prior art, and any objective evidence of non-obviousness such as long-felt need, and commercial success.

Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > Infringement Actions > Defenses > Patent Invalidity > Validity Presumption
Patent Law > Nonobviousness > Evidence & Procedure > Presumptions & Proof
[HN7] Because an issued patent is presumed valid, there must be clear and convincing evidence supporting the obviousness determination.

Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > Nonobviousness > Evidence & Procedure > General Overview
Patent Law > U.S. Patent & Trademark Office Proceedings > General Overview
[HN8] In appropriate circumstances, a single prior art reference can render a claim obvious. However, there must be a showing of a suggestion or motivation to modify the teachings of that reference to the claimed invention in order to support the obviousness conclusion. This suggestion or motivation may be derived from the prior art reference itself, from the knowledge of one of ordinary skill in the art, or from the nature of the problem to be solved.

Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > Nonobviousness > Elements & Tests > Prior Art
Patent Law > Nonobviousness > Evidence & Procedure > Fact & Law Issues
[HN9] In patent law, determining whether there is a suggestion or motivation to modify a prior art reference is one aspect of determining the scope and content of the prior art, a fact question subsidiary to the ultimate conclusion of obviousness

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN10] In patent law, when the record establishes a strong case of obviousness based on the teachings of the prior art, the fact that the product was successful does not overcome the conclusion of obviousness.

Civil Procedure > Appeals > Standards of Review > Substantial Evidence > Sufficiency of Evidence
Patent Law > Infringement Actions > Burdens of Proof
[HN11] In patent law, for objective evidence to be accorded substantial weight, its proponent must establish a nexus between the evidence and the merits of the claimed invention.

Patent Law > Claims & Specifications > Claim Language > Representative Claims
Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > Nonobviousness > Evidence & Procedure > Fact & Law Issues
[HN12] In patent law, dependent claims fall with the independent claim on which they depend unless argued separately.

COUNSEL: Stephen P. Swinton, Cooley Godward LLP, of San Diego, California, argued for plaintiff-appellee. With him on the brief were Anthony M. Stiegler, J. Christopher Jaczko, Kent M. Walker, and Amy S. Hellenkamp.

Laura A. Coruzzi, Pennie & Edmonds LLP, of New York, New York, argued for defendant-appellant. With her on the brief was S. Leslie Misrock. Of counsel on the brief were Stanton T. Lawrence, III, Paul J. Zegger, and Carl P. Bretscher, Pennie & Edmonds LLP, of Washington, DC.

JUDGES: Before MAYER, Chief Judge, MICHEL and GAJARSA, Circuit Judges. Opinion for the court filed by Circuit Judge Gajarsa. Chief Judge Mayer dissents.

OPINION BY: GAJARSA

OPINION

[*1351] GAJARSA, *Circuit Judge.*

Cadus Pharmaceutical Corporation ("Cadus") appeals the judgment of the United States District Court for the Southern District of California entered after a jury verdict finding the patent claims at issue infringed and not invalid, and assessing damages of $ 18 million. Because we determine that the asserted claims are obvious as a matter of law, we reverse.

BACKGROUND

The identification [**2] of compounds that bind with particular cell surface proteins is useful in the search for new drugs. When such binding occurs, a cascade of biochemical events is activated within the cell in which a linkage, known as a signal transduction pathway, is formed between the cell surface protein and a gene in the cell's DNA. This linkage allows the cell to respond to signals from the external environment, which is critical for the cell to properly function. Compounds that activate this linkage often prove useful in pharmaceutical applications because many diseases stem from the malfunctioning of cellular communications.

[*1352] In general, when a compound activates a signal transduction pathway, the cell responds by directing the production or non-production of a protein from a responsive gene in the DNA. Protein production involves two distinct processes--transcription and translation. Transcription refers to the process by which a strand of messenger RNA ("mRNA") is created by the expression of a gene. Translation refers to the process by which a corresponding protein (i.e., a sequence of amino acids) is created from the mRNA. Compounds that trigger or enhance transcription and translation are [**3] referred to as agonists, and compounds that block or decrease such activity are called antagonists. The displaying of agonist and antagonist activity is an indication that a compound has bound with the cell surface protein and has activated the signal transduction pathway.

SIBIA Neurosciences, Inc. ("SIBIA") is the owner of *U.S. Patent No. 5,401,629 ("the '629 patent")*, which is directed to a cell-based screening method useful for the identification of compounds that exhibit agonist and antagonist activity with respect to particular cell surface proteins. According to the patent, the claimed methods are particularly effective because they allow a scientist to rapidly and reliably screen large numbers of compounds for agonist and antagonist activity. *See '629 patent*, col. 1, ll. 45-50. Thus, the scientist could quickly develop a list of candidate compounds that merit further in-depth studies for therapeutic applications. *See id.* Claim 1, the only independent claim, reads as follows:

> 1. A method for identifying compounds that modulate cell surface protein-mediated activity by detecting intracellular transduction of a signal generated upon interaction of the compound [**4] with the cell surface protein, comprising:
>
> comparing the amount of transcription of a reporter gene or the amount of reporter gene product expressed in a first recombinant cell in the presence of the compound with the amount of transcription or product in the absence of the compound, or with the amount of transcription or product in a second recombinant cell; and

> selecting compounds that change the amount of transcription of a reporter gene or the amount of reporter gene product expressed in the first recombinant cell in the presence of the compound compared to the amount of transcription or product in the absence of the compound, or compared to the amount of transcription or product in the second recombinant cell, wherein:
>
> the cell surface protein is a surface receptor or ion channel;
>
> the first recombinant cell contains a reporter gene construct and expresses the cell surface protein;
>
> the second recombinant cell is identical to the first recombinant cell, except that it does not express the cell surface protein; and
>
> the reporter gene construct contains:
>
> (a) a transcriptional control element that is responsive to the intracellular signal that is generated by the interaction [**5] of an agonist with the cell surface protein; and
>
> (b) a reporter gene that encodes a detectable transcriptional or translational product and that is in operative association with the transcriptional control element.

See id., col. 13, l. 44 - col. 14, l. 12.

The methods claimed in the *'629 patent* utilize a recombinant cell that is exposed to various compounds in order to determine whether those compounds exhibit the desired activity. This recombinant cell, in addition to the host cell itself, has two basic components: a heterologous [1] cell surface protein and a reporter gene construct. The cell surface protein can be either an ion channel or a cell surface receptor. Ion channels are proteins that act as pores in the cell membrane and allow small inorganic ions to flow in or out [*1353] of the cell. These ion channels open and close based on interaction with certain external compounds. Cell surface receptors, on the other hand, are proteins that span the external membrane of the cell and bind with particular molecules to commence a chain of intracellular reactions that transmit external signals to the DNA. As described above, cell surface proteins are physiologically important [**6] because they play a vital role in the stimulation of signal transduction pathways, and thus, the cell's ability to respond appropriately to stimuli from the external environment.

> 1 A cell surface protein is "heterologous" if it is not naturally occurring in the cell.

The second major component of the cell utilized in the *'629 patent* is the reporter gene construct, which consists of a transcriptional control element and a reporter gene. The transcriptional control element is a gene that reacts to the signal from the cell surface protein and regulates transcription of the reporter gene. The reporter gene, through the processes of transcription and translation, creates a corresponding protein, referred to as "reporter gene product." Both transcription of the reporter gene and translation to the reporter gene product can be measured.

In the claimed methods, this recombinant cell is used in a battery of assays, the goal of which is to determine if a given compound exhibits the desired binding activity with respect [**7] to a particular cell surface protein. The method of claim 1 contains two assays. In the first assay, referred to as the "compound/no compound assay," the recombinant cell is exposed to a test compound. The amount of reporter gene transcription, or reporter gene product expressed in that recombinant cell, is then compared to the amount of reporter gene transcription or reporter gene product expressed in a recombinant cell that was not exposed to the test compound. In the second assay, known as the "receptor/no receptor assay," two recombinant cells are exposed to a test compound. However, one of the recombinant cells has a cell surface protein, but the other does not. The amount of reporter gene transcription or reporter gene product expressed in both of these cells is then compared. Based on these measurements, the scientist is able to detect whether the compound has bound to the cell surface protein and modulated the signal transduction pathway. This, in turn, allows the scientist to determine whether the compound is a candidate for further study, or should be excluded from consideration.

SIBIA sued Cadus for infringement of the *'629 patent*. The court held a *Markman* hearing, [**8] *see Markman v. Westview Instruments, Inc., 52 F.3d 967, 979, 34 U.S.P.Q.2D (BNA) 1321, 1329 (Fed. Cir. 1995)* (en banc), *aff'd, 517 U.S. 370, 134 L. Ed. 2d 577, 116 S. Ct. 1384 (1996)*, and issued an order construing numerous claim terms, including "identifying compounds," "cell," "recombinant cell," "comparing the amount," "identical" and "selecting compounds." Only the construction of "cell," "identifying compounds," and "selecting compounds" are relevant to this appeal. Before the district court, Cadus argued that because the claims use the term "cell" without modification, this term should refer to all cells, eukaryotic as well as prokaryotic. [2] Al-

ternatively, Cadus argued that if "cell" should be limited to less than *all* cells, it should be limited to only mammalian cells, because the examples found in the written description of the patent only discuss mammalian cells. The court decided, however, that because the patentee describes the cells used in the claimed methods as "eukaryotic cells" in the written description, *see '629 patent*, col. 3, ll. 52-56, col. 4, ll. 9-11, a person of ordinary skill in the art would interpret cell as found in the claim [**9] language to mean only eukaryotic cells.

> 2 Eukaryotic cells, such as animal, plant, yeast, and fungal cells, have nuclei where the cell's genetic material is contained. Prokaryotic cells, such as bacteria and blue-green algae cells, do not have nuclei.

Also important to this appeal is the court's construction of the phrases "identifying compounds" and "selecting compounds." [*1354] At the *Markman* hearing, the parties disagreed as to whether this claim language required the compounds to be unknown to interact with the particular cell surface protein prior to conducting the assays, or whether these terms include both compounds known and unknown to interact with the cell surface proteins. The court held that the ordinary meaning of "identifying compounds" is determining which compounds interact with a particular cell surface protein from a group of compounds with unknown properties. Thus, the testing of compounds that are known to interact with a particular cell surface protein does not fall within the ambit of "identifying [**10] compounds." Similarly, the court held that "selecting compounds" referred only to choosing compounds from a group previously unknown to interact with a cell surface protein based on the results of the reporter gene transcription and translation analyses.

The case then proceeded to a jury trial. At trial, Cadus asserted that the claims of the *'629 patent* were invalid as obvious under *35 U.S.C. § 103(a)* or as not enabled under *§ 112*, P 1. With regard to obviousness, Cadus asserted that the claims of the *'629 patent* would have been obvious in view of Deborah J. Stumpo et al., *Identification of c-fos Sequences Involved in Induction by Insulin and Phorbol Esters*, 263 J. Biological Chem. 1611 (Feb. 1988) ("Stumpo") alone, given the knowledge in the art as embodied in the review article by Henry A. Lester, Heterologous Expression of Excitability Proteins: Route to More Specific Drugs?, 241 Science 1057 (Aug. 1988) ("Lester"). Additionally, Cadus asserted that the claims would have been obvious in view of Stumpo in combination with William S. Chen et al., *Requirement for intrinsic protein tyrosine kinase in the immediate and late action of the EGF receptor*, [**11] 328 Nature 820 (Aug. 1987) ("Chen"), and Ronald Mark Evans et al., *Hormone Receptor Compositions and Methods*, WO 88/03168 (May 1988) ("Evans"). With regard to non-enablement, Cadus claimed that if "cell" is to be interpreted to broadly include all eukaryotic cells, the claims are not enabled because the written description discloses only how to practice the invention using mammalian cells. The jury returned a verdict in favor of SIBIA, finding that Cadus infringed claims 1, 2, 4-7, 9, 10, 12, and 14 of the *'629 patent*. The jury rejected Cadus's invalidity defenses of obviousness and non-enablement. Damages, based on the calculation of a "reasonable royalty," were assessed at $ 18 million. Cadus filed numerous post-trial motions, including motions for judgment as a matter of law ("JMOL") or a new trial on the issues of infringement and invalidity, and motions for remittitur or a new trial for damages. All of Cadus's motions were denied. This appeal followed.

DISCUSSION

A. Standard of Review

[HN1] We review the denial of a motion for JMOL following a jury verdict by reapplying the district court's standard of review. *See Tec Air, Inc. v. Denso Mfg., 192 F.3d 1353, 1357, 52 U.S.P.Q.2D (BNA) 1294, 1296 (Fed. Cir. 1999).* [**12] Thus, we review issues of law *de novo*. With regard to factual findings, we must presume that the jury resolved all factual disputes in favor of the prevailing party, and we must leave those findings undisturbed as long as they are supported by substantial evidence. *See Jurgens v. McKasy, 927 F.2d 1552, 1557, 18 U.S.P.Q.2D (BNA) 1031, 1035 (Fed. Cir. 1991).*

[HN2] A factual finding is supported by substantial evidence if a reasonable jury could have found in favor of the prevailing party in light of the evidence presented at trial. *See Tec Air, 192 F.3d at 1358, 52 U.S.P.Q.2D (BNA) at 1296; see also Consolidated Edison Co. v. NLRB, 305 U.S. 197, 229, 83 L. Ed. 126, 59 S. Ct. 206 (1938)* ("Substantial evidence is more than a mere scintilla. It means such relevant evidence as a reasonable mind might accept as adequate to support a conclusion."). Thus, substantial evidence review involves an examination of the record as a whole, taking into consideration [*1355] evidence that both justifies and detracts from the decision of the fact-finder. *See In re Gartside, 203 F.3d 1305, 1312, 53 U.S.P.Q.2D (BNA) 1769, 1773 (Fed. Cir. 2000); National Presto Indus., Inc. v. West Bend Co., 76 F.3d 1185, 1192, 37 U.S.P.Q.2D (BNA) 1685, 1690 (Fed. Cir. 1996)* [**13] (holding that a jury verdict must be sustained if it is supported by substantial evidence based on a review of the entirety of the record). [HN3] In reviewing the record, we must draw all reasonable inferences in favor of the prevailing party, and not make credibility determinations or substitute our view of the conflicting evidence for that of the jury. *See Connell*

v. Sears, Roebuck & Co., 722 F.2d 1542, 1546, 220 U.S.P.Q. (BNA) 193, 197 (Fed. Cir. 1983). If, however, after reviewing all of the evidence in a light most favorable to the prevailing party, this court is convinced that a reasonable jury could not have found in that party's favor, we must reverse the denial of JMOL.

B. Obviousness

[HN4] The first step in any invalidity analysis is claim construction, an issue of law that this court reviews *de novo. See Cybor Corp. v. FAS Techs., Inc., 138 F.3d 1448, 1456, 46 U.S.P.Q.2D (BNA) 1169, 1174 (Fed. Cir. 1998)* (en banc). In this appeal, the key issues of claim construction are largely undisputed. As described in more detail above, the method of claim 1 utilizes a recombinant cell having both a heterologous cell surface protein and a reporter gene construct. This cell is [**14] used in two assays--the compound/no compound assay and the receptor/no receptor assay--in which compounds are "identified" and "selected." Neither party disputes that the terms "identifying compounds" and "selecting compounds" limit the claimed method to identifying and selecting compounds that are not previously known to interact with a particular cell surface protein. The only remaining claim construction issue on appeal is the proper interpretation of the term "cell." According to Cadus, the court erred by limiting "cell" to only eukaryotic cells, as opposed to all cells, both eukaryotic and prokaryotic. SIBIA defends the district court's interpretation by pointing to certain passages in the written description that, it asserts, support the district court's narrower claim construction. *See Comark Communications, Inc. v. Harris Corp., 156 F.3d 1182, 1186, 48 U.S.P.Q.2D (BNA) 1001, 1005 (Fed. Cir. 1998)* (discussing the "fine line" between reading a claim in light of the written description and reading a limitation into the claim from the written description). However, because we decide that the claim is obvious even under the district court's narrow construction of the term [**15] "cell," we need not decide whether the court erroneously imported the "eukaryotic" limitation into the claim, or simply interpreted the claim in light of the specification. Thus, we can proceed to the question of obviousness accepting the district court's construction of claim 1.

[HN5] A patent claim is invalid "if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art." *35 U.S.C. § 103(a) (1994).* [HN6] While the ultimate conclusion of obviousness is for the court to decide as a matter of law, several factual inquiries underlie this determination. *See Graham v. John Deere Co., 383 U.S. 1, 17-18, 15 L. Ed. 2d 545, 86 S. Ct. 684 (1966).* These inquiries include the scope and content of the prior art, the level of ordinary skill in the field of the invention, the differences between the claimed invention and the prior art, and any objective evidence of non-obviousness such as long-felt need, and commercial success. *See id.* [HN7] Because an issued patent is presumed valid, there must be [**16] clear and convincing evidence supporting the obviousness determination. *See Kahn v. General Motors Corp., 135 F.3d 1472, 1480, 45 U.S.P.Q.2D (BNA) 1608, 1614 (Fed. Cir. 1998).* While the presentation at trial of a reference that was not before the examiner does not change the presumption of validity, the alleged infringer's burden [*1356] may be more easily carried because of this additional reference. *See Applied Materials, Inc. v. Advanced Semiconductor Materials Am., Inc., 98 F.3d 1563, 1569, 40 U.S.P.Q.2D (BNA) 1481, 1485 (Fed. Cir. 1996).*

On appeal, Cadus argues that the Stumpo reference alone, which was not before the PTO examiner, is sufficient to invalidate the patent under *§ 103*, given the level of skill in the art at the time of the invention. [HN8] In appropriate circumstances, a single prior art reference can render a claim obvious. *See, e.g., B.F. Goodrich Co. v. Aircraft Braking Sys. Corp., 72 F.3d 1577, 1582, 37 U.S.P.Q.2D (BNA) 1314, 1318 (Fed. Cir. 1996); In re O'Farrell, 853 F.2d 894, 902, 7 U.S.P.Q.2D (BNA) 1673, 1680 (Fed. Cir. 1988).* However, there must be a showing of a suggestion or motivation to modify the teachings of that reference to the claimed [**17] invention in order to support the obviousness conclusion. *See B.F. Goodrich, 72 F.3d at 1582, 37 U.S.P.Q.2D (BNA) at 1318.* This suggestion or motivation may be derived from the prior art reference itself, *see O'Farrell, 853 F.2d at 902, 7 U.S.P.Q.2D (BNA) at 1680,* from the knowledge of one of ordinary skill in the art, or from the nature of the problem to be solved. *See Pro-Mold & Tool Co. v. Great Lakes Plastics, Inc., 75 F.3d 1568, 1573, 37 U.S.P.Q.2D (BNA) 1626, 1630 (Fed. Cir. 1996); see also Motorola, Inc. v. Interdigital Tech. Corp., 121 F.3d 1461, 1472, 43 U.S.P.Q.2D (BNA) 1481, 1489 (Fed. Cir. 1997)* ("The suggestion to combine may come from the prior art, as filtered through the knowledge of one skilled in the art."). [HN9] Determining whether there is a suggestion or motivation to modify a prior art reference is one aspect of determining the scope and content of the prior art, a fact question subsidiary to the ultimate conclusion of obviousness. *See Tec-Air, Inc., 192 F.3d at 1359, 52 U.S.P.Q.2D (BNA) at 1298* (stating that the factual underpinnings of obviousness include whether a reference provides a motivation to combine its teachings with another). [**18] Because the jury returned a verdict in favor of SIBIA, we must presume that all factual disputes, such as the motivation to modify, were resolved in its favor. *See Jurgens, 927 F.2d at 1557, 18 U.S.P.Q.2D (BNA) at 1035.*

The parties are in general agreement regarding the teachings of the Stumpo paper itself. Stumpo describes recombinant cells engineered to have both a heterologous cell surface receptor and a responsive reporter gene construct. These cells are identical to the recombinant cells used in the claimed methods. Stumpo describes using these cells in a transcription-based assay in order to detect cell surface receptor activation. According to the unrebutted testimony of Dr. Struhl, the Stumpo paper described a "straightforward functional assay" for analyzing the response of a particular cell surface protein in the presence of a compound. However, these transcription-based assays use the compound insulin, which was known to interact with the surface receptors of Stumpo's recombinant cells. The purpose of these assays was not drug screening, but the characterization of certain aspects of the genetic material of the recombinant cell. Claim 1 of the *'629 patent*, on the [**19] other hand, claims a method using recombinant cells identical to Stumpo's in transcription-based assays with compounds not previously known to interact with the cell surface protein of the recombinant cell. The only difference between the experiments described in the Stumpo paper and claim 1 is that in the Stumpo paper, the compounds are known to interact with the cell surface proteins, while in claim 1, they are not. Thus, we must presume that the jury determined that there was no motivation to modify the Stumpo reference such that the cells described therein would be utilized with compounds that were not previously known to interact with the cell surface proteins. *See id.* We hold that this key factual finding is not supported by substantial evidence.

As discussed above, the motivation to modify a reference can come from the knowledge of those skilled in the art, from the prior art reference itself, or from the nature of the problem to be solved. *See In re Rouffet, 149 F.3d 1350, 1358, 47* [*1357] *U.S.P.Q.2D (BNA) 1453, 1458 (Fed. Cir. 1998).* The undisputed evidence indicates that there was a motivation to modify Stumpo. It was known in the art at the time of the invention that [**20] cells with heterologous cell surface proteins were ideal candidates for drug screening methods. The Lester review article describes the widespread use of such cells in the identification of new drugs:

> A new approach for a systematic program to develop more specific drugs has simultaneously occurred to several investigators. This approach is based on the expression of excitability molecules [3] from DNA clones in cells that readily support such expression and can readily be studied with the full range of modern physiological and pharmacological techniques.

Lester, 241 Science at 1058. Lester goes on to describe that drug screening methods utilizing the expression of excitability molecules (i.e., cell surface receptors) can overcome the "highly empirical approach to the design of drugs" and the lack of functional assays for determining which compounds act on which cell surface receptors. *Id. at 1062.* These are the identical problems that were being addressed by the *'629 patent. See '629 patent*, col. 1, ll. 36-44 ("The availability of rapid, effective means to identify compounds which interact with . . . cell surface-localized receptors would enable the rapid screening [**21] of a large number of compounds to identify those candidates suitable for further in-depth studies of therapeutic applications."). Similarly, the prior art Dull patent (*U.S. Patent No. 4,859,609*) teaches a drug screening method using cells that had a cell surface receptor. Thus, the express teaching in the prior art was that cells having heterologous cell surface proteins, a characteristic found in the Stumpo cells, have been successfully used in drug screening methods and were, in fact, ideal candidates for such use. Additionally, the undisputed testimony was that Stumpo provided a "straightforward functional assay" for determining the response of the heterologous cell surface protein when exposed to a compound. Given that the nature of the problem was the development of rapid and effective drug screening methods based on the response of a heterologous cell surface protein, these teachings provide the motivation to modify Stumpo.

3 The "excitability molecules" referred to in Lester are identical to the "cell surface proteins" referred to in the *'629 patent*.

[**22] In response to these teachings, SIBIA merely points out that the cells described in the Lester article and the Dull patent are not described as having reporter gene constructs like those used in the *'629 patent* and found in the Stumpo cells. SIBIA, however, is confusing obviousness with anticipation. It is true that these references do not contain an express teaching to use a cell identical to that taught by Stumpo in a drug screening method. It is equally true that these references, particularly Lester, teach that cells with heterologous cell surface receptors were known in the art to have been successfully used in drug screening methods and that the Stumpo cells have such heterologous cell surface receptors. SIBIA's response, that Lester does not mention cells that contain a reporter gene construct in addition to the heterologous cell surface receptor, is to no avail absent some evidence that this additional characteristic would have made such a cell a less attractive candidate for drug screening methods. *See In re Gurley, 27 F.3d 551, 553, 31 U.S.P.Q.2D (BNA) 1130, 1131 (Fed. Cir. 1994)* ("[A] reference will teach away if it suggests that the line of

development flowing [**23] from the reference's disclosures is unlikely to be productive of the result sought by the applicant."). SIBIA makes no allegation of a teaching away in Lester. To the contrary, the evidence is unrebutted that cells with reporter gene constructs were also known in the art to be useful in drug screening methods. *See U.S. Patent No. 5,091,518* to Sucov. [4] [*1358] Thus, these undisputed teachings in the prior art, "as filtered through the knowledge of one skilled in the art," *Motorola, 121 F.3d at 1472, 43 U.S.P.Q.2D (BNA) at 1489*, as well as the nature of the problem to be solved, provide a suggestion and motivation to use the Stumpo cells, which have heterologous cell surface receptors, in drug screening methods.

4 Contrary to the suggestion in the dissenting opinion, Sucov is not being "combined" with Stumpo or Lester to achieve the obviousness conclusion. Rather, Sucov is merely cited as an example showing that the use of cells with reporter gene constructs was known in the art to be useful in drug screening methods.

[**24] SIBIA asserts that, regardless of these express teachings supporting the suggestion to modify Stumpo, various trial testimony provides the substantial evidence on which the jury's implied finding of no motivation to modify can be supported. SIBIA relies heavily on the testimony of Drs. Wall, Struhl, and Blackshear. Dr. Wall testified that the Stumpo paper contained no mention of drug screening, and that the experiments described in that paper were directed to the characterization of the *fos* gene, not to a drug screening method. Wall also testified that the Stumpo paper would not immediately lead one to "conduct drug screening." Similarly, Dr. Struhl testified that there was no indication that the researchers involved in the experiments described in Stumpo used the cells for drug screening. However, simply pointing out that the Stumpo reference itself does not teach the modification is not substantial evidence of no motivation to modify, given the express teaching of the prior art. SIBIA's reliance on the testimony of Wall and Struhl ignores the possibility that the motivation to modify Stumpo can be found outside the reference itself. *See id.* Thus, while Stumpo does not expressly [**25] suggest that the cells described therein could be used in drug screening methods, the knowledge of those skilled in the art, in particular as embodied in the Lester review article, suggests this modification. SIBIA also points to the testimony of Dr. Blackshear, the senior author of Stumpo, who testified that the Stumpo paper does not contain any reference to drug screening, and at the time those experiments were conducted, "drug screening was not on our minds." However, this testimony, in itself, does not provide substantial evidence in support of the jury's finding. At the time of these experiments, Blackshear was focused on the problem of determining the "fundamental biochemical mechanisms by which insulin worked." Blackshear's personal efforts were limited to a problem different than that addressed by the *'629 patent*. Thus, the testimony that he was not thinking about drug screening is irrelevant to the fundamental issue of whether the hypothetical person of ordinary skill in the art, when confronted with the problem of developing drug screening methods, would have been motivated to use the Stumpo cells in such methods. *See Pro-Mold & Tool Co., 75 F.3d at 1573, 37 U.S.P.Q.2D (BNA) at 1630* [**26] (discussing the importance of considering the problem to be solved in the obviousness determination); *see also In re Rinehart, 531 F.2d 1048, 1054, 189 U.S.P.Q. (BNA) 143, 149 (CCPA 1976)* (same).

Finally, SIBIA points to secondary considerations in support of the jury's verdict. In particular, SIBIA points to three licenses or sub-licenses of the *'629 patent*, all of which were part of larger licensing packages. However, the mere existence of these licenses is insufficient to overcome the conclusion of obviousness, as based on the express teachings in the prior art that would have motivated one of ordinary skill to modify Stumpo's cells to be used with unknown compounds. *See Newell Cos. v. Kenney Mfg. Co., 864 F.2d 757, 769, 9 U.S.P.Q.2D (BNA) 1417, 1426 (Fed. Cir. 1988)* (holding that [HN10] because the record established such a strong case of obviousness based on the teachings of the prior art, the fact that the product was successful does not overcome the conclusion of obviousness). Moreover, SIBIA has failed to point to any evidence establishing a nexus between the licensing activity and the merits of the claimed screening method. *See* [*1359] *In re GPAC Inc., 57 F.3d 1573, 1580, 35 U.S.P.Q.2D (BNA) 1116, 1121 (Fed. Cir. 1995)* [**27] ("[HN11] For objective evidence to be accorded substantial weight, its proponent must establish a nexus between the evidence and the merits of the claimed invention."). Thus, SIBIA's reliance on secondary considerations in support of the jury verdict must fail.

In sum, the undisputed teaching of the Stumpo paper leads one to within a hairsbreadth of anticipation of claim 1 of the *'629 patent*. The express teachings in the art provide the motivation and suggestion to modify Stumpo such that the recombinant cells described therein should be used with compounds not previously known to interact with them for purposes of drug screening. SIBIA, the jury verdict winner, has failed to point to any substantial evidence to refute these express teachings, even under the deferential standard with which this court reviews jury verdicts. Thus, claim 1 must be invalidated on the basis of obviousness.

C. Dependent Claims

In addition to finding claim 1 infringed and not invalid, the jury found dependent claims 2, 4-7, 9, 10, 12, and 14 infringed and not invalid as well. However, in this appeal, SIBIA has failed to argue the validity of the dependent claims separately from the validity of claim 1. Thus, [**28] these claims do not stand on their own, and given our determination that claim 1 is invalid, the remaining dependent claims must fall as well. *See Mehl/Biophile Int'l Corp. v. Milgraum, 192 F.3d 1362, 1367, 52 U.S.P.Q.2D (BNA) 1303, 1307 (Fed. Cir. 1999); Gardner v. Tec Sys. Inc., 725 F.2d 1338, 1350, 220 U.S.P.Q. (BNA) 777, 786 (Fed. Cir. 1984)* (en banc) (holding that [HN12] dependent claims fall with the independent claim on which they depend unless argued separately).

CONCLUSION

We conclude that the implicit finding by the jury that there was no suggestion or motivation to modify the Stumpo reference is unsupported by substantial evidence and the asserted claims are obvious as a matter of law. Therefore, the district court's denial of the Cadus's motion for JMOL on the issue of invalidity must be

REVERSED.

COSTS

Each party shall bear its own costs.

DISSENT BY: MAYER

DISSENT

MAYER, *Chief Judge*, dissenting.

Today, the court overrides a jury verdict of infringement based on a tenuous obviousness analysis. It recognizes that the Stumpo paper only refers to the use of known substances and presumes that, to find infringement, the jury must have implicitly found that [**29] there was no motivation in Stumpo to utilize the disclosed cells with compounds not previously known to interact with the cell surface proteins. Based on the state of knowledge in the art that cells with heterologous cell surface proteins were ideal candidates for drug screening methods, the court then concludes that the jury's implicit finding is not supported by substantial evidence.

In reality, the court relies on the combination of Stumpo, Lester, and Sucov to establish that the use of heterologous cells with reporter gene constructs was known in the art to be useful in drug screening methods. Stumpo discloses cells identical to the *'629 patent* claims, but does not mention their use to test unknown compounds as possible drugs. Lester describes the utility of heterologous cell surface proteins for drug testing, but does not mention cells with reporter gene constructs, which are central to the method of testing of the '629 claims. Sucov was not even argued at trial, where Cadus argued that either Stumpo or Chen renders the *'629 patent* obvious. This analysis is inconsistent with the court's stated conclusion that the *'629 patent* is obvious over the Stumpo reference alone in view [**30] of the prior art as argued by Cadus. It fails to recognize that the *'629 patent* includes only method claims; Sibia disclaimed all claims [*1360] to the cells themselves when Stumpo was brought to its attention.

The court is making an end-run around the requirement that there must be a motivation to modify the reference along the path taken by the *'629 patent. See Kolmes v. World Fibers Corp., 107 F.3d 1534, 1541, 41 U.S.P.Q.2D (BNA) 1829, 1833 (Fed. Cir. 1997)* (Invention was not obvious where there was no suggestion or motivation to modify teaching of reference.). It combines a series of references not specifically argued to the jury to conclude that no reasonable jury could possibly find the *absence* of motivation in the prior art to modify the Stumpo paper to render the *'629 patent* obvious. Without citing any motivation to modify in any of the series of references, the court improperly concludes that it would have been unreasonable for the jury to find as a matter of fact that there was no such motivation. *See Tec Air, Inc. v. Denso Mfg. Michigan, Inc., 192 F.3d 1353, 1359, 52 U.S.P.Q.2D (BNA) 1294, 1297-98 (Fed. Cir. 1999)* (Whether a reference provides a motivation to [**31] combine its teachings with other references is a question of fact underlying the legal determination of nonobviousness that we assume the jury resolved in favor of the verdict winner and leave undisturbed if it is supported by substantial evidence.).

The district court properly rejected Cadus' motion for judgment as a matter of law, holding that there was substantial evidence to support a verdict of nonobviousness because the *'629 patent* was a "combination of factors that was not apparent to a person of ordinary skill in the art." The trial court found additional support for the jury's verdict in evidence of secondary considerations of long-felt need and commercial success of the *'629 patent.* These are factual underpinnings of the legal conclusion of nonobviousness that the jury presumptively resolved in favor of Sibia because substantial evidence supported them. *See id., 52 U.S.P.Q.2D (BNA) at 1298.*

This court improperly rejects this substantial evidence. It opens the door for accused infringers to string together a series of references, which collectively contain the elements of an apparatus (here, the cell with a heterologous cell surface protein and reporter gene construct) [**32] and various suggestions for the use of those separate references. It then would allow an inference of motivation to modify a single reference to render obvious a method claim for utilizing the apparatus. All

this is in violation of the well-settled mandate requiring a motivation to alter a single reference or to combine multiple references to render the claims of a patent obvious. *See, e.g., id. at 1359, 52 U.S.P.Q.2D (BNA) at 1298* (motivation to combine multiple references); *B.F. Goodrich v. Aircraft Braking Sys. Corp., 72 F.3d 1577, 1582, 37 U.S.P.Q.2D (BNA) 1314, 1318 (Fed. Cir. 1996)* (motivation to modify a single reference); *Grain Processing Corp. v. American Maize-Products Co., 840 F.2d 902, 907, 5 U.S.P.Q.2D (BNA) 1788, 1792 (Fed. Cir. 1988)* ("Care must be taken to avoid hindsight reconstruction by using 'the patent in suit as a guide through the maze of prior art references, combining the right references in the right way so as to achieve the result of the claims in suit.'") (internal citation omitted); *In re Fine, 837 F.2d 1071, 1075, 5 U.S.P.Q.2D (BNA) 1596, 1600 (Fed. Cir. 1988)* ("One cannot use hindsight reconstruction to pick and choose [**33] among isolated disclosures in the prior art to deprecate the claimed invention."). The court has substituted itself for the jury, reweighed the evidence, and combined references that were not before the jury. I would sustain the jury's verdict.

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U.S. Appln. No. 10/770,639
Reference No. 16

LEXSEE 391 F.3D 1195

IN RE DANIEL S. FULTON and JAMES HUANG

04-1267

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

391 F.3d 1195; 2004 U.S. App. LEXIS 24815; 73 U.S.P.Q.2D (BNA) 1141

December 2, 2004, Decided

PRIOR HISTORY: [**1] Appealed from: United States Patent and Trademark Office Board of Patent Appeals and Interferences. (Serial No. 09/122,198).

DISPOSITION: Affirmed.

CASE SUMMARY:

PROCEDURAL POSTURE: Appellant patent applicants sought review of a decision of the United States Patent and Trademark Office Board of Patent Appeals and Interferences, which affirmed a hearing examiner's rejection of their application for a utility patent for a shoe sole with increased traction on grounds that the invention would have been obvious under *35 U.S.C.S. § 103(a)*.

OVERVIEW: The claim limitations were that (1) the shoe perimeter was mostly open, (2) the projected surfaces were hexagonal, and (3) the hexagons had a facing orientation. The Board found that prior art suggested a combination of the open perimeter with the hexagonal surface and facing orientation. The court affirmed. The Board's findings were supported by substantial evidence. Under *§ 103(a)*, when a patent rejection depended on a combination of prior art references, some teaching or motivation to combine the references was required. However, a particular combination did not have to be the preferred one. The Board's secondary findings supported a primary finding that the prior art suggested the desirability of this combination. The Board properly found that prior art references taught away from the combination. Mere disclosure of alternative designs did not teach away. Prior art disclosed a number of alternative shoe sole designs, but did not teach that hexagonal projections in a facing orientation were undesirable. The Board's findings encompassed the use of a facing orientation. The Board's analysis contained sufficient comparative reasoning and provided a factual basis for review.

OUTCOME: The court affirmed the decision.

LexisNexis(R) Headnotes

Patent Law > Nonobviousness > Elements & Tests > Claimed Invention as a Whole
Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN1] See *35 U.S.C.S. § 103(a)*.

Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN2] Obviousness is a question of law based on underlying findings of fact. The United States Patent and Trademark Office Board of Patent Appeals and Interferences' factual findings are upheld unless they are unsupported by substantial evidence. Substantial evidence is such relevant evidence as a reasonable mind might accept as adequate to support a conclusion. What the prior art teaches, whether it teaches away from the claimed invention, and whether it motivates a combination of teachings from different references are questions of fact. Other factual findings related to obviousness may include (1) the scope and content of the prior art; (2) the level of ordinary skill in the prior art; (3) the differences between the claimed invention and the prior art; and (4) objective evidence of nonobviousness.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview

Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > Nonobviousness > Elements & Tests > Teaching Away From Invention
[HN3] When a patent rejection depends on a combination of prior art references, there must be some teaching, suggestion, or motivation to combine the references. Stated another way, the prior art as a whole must "suggest the desirability" of the combination. Trade-offs often concern what is feasible, not what is, on balance, desirable. Motivation to combine requires the latter. The source of the teaching, suggestion, or motivation may be "the nature of the problem," "the teachings of the pertinent references," or "the ordinary knowledge of those skilled in the art."

Patent Law > Nonobviousness > Elements & Tests > Claimed Invention as a Whole
Patent Law > Nonobviousness > Elements & Tests > Prior Art
Patent Law > Nonobviousness > Evidence & Procedure > General Overview
[HN4] Case law does not require that a particular combination must be the preferred, or the most desirable, combination described in the prior art in order to provide motivation for the current invention. The question is whether there is something in the prior art as a whole to suggest the desirability, and thus the obviousness, of making the combination, not whether there is something in the prior art as a whole to suggest that the combination is the most desirable combination available.

Patent Law > Nonobviousness > Elements & Tests > Teaching Away From Invention
Patent Law > Nonobviousness > Evidence & Procedure > General Overview
[HN5] As long as some motivation or suggestion to combine the references is provided by the prior art taken as a whole, the law does not require that the references be combined for the reasons contemplated by the inventor.

COUNSEL: Garth E. Janke, Birdwell & Janke, of Portland, Oregon, for appellants.

John M. Whealan, Solicitor, United States Patent and Trademark Office, of Arlington, Virginia, for the Director of the United States Patent and Trademark Office. With him on the brief were W. Asa Hutchinson III, Attorney-Advisor, and William LaMarca, Associate Solicitor.

JUDGES: Before MICHEL, RADER, and GAJARSA, Circuit Judges.

OPINION BY: MICHEL

OPINION

[*1196] MICHEL, *Circuit Judge*.

Appellants Daniel Fulton and James Huang appeal from the decision of the U.S. Patent and Trademark Office, Board of Patent Appeals and Interferences ("Board"), affirming the examiner's rejection of appellants' application for a utility patent on grounds that the invention claimed would have been obvious under *35 U.S.C. § 103(a)*. The appeal was submitted for decision without oral argument on November 5, 2004. Because the Board's finding that the prior art suggested the desirability of the combination of shoe sole limitations claimed in appellants' patent application was supported [**2] by substantial evidence, we affirm.

Background

On July 24, 1997, appellants filed application number 09/122,198 (the "'198 application") for a utility patent directed to a shoe sole with increased traction. Claim 1, the only independent claim at issue, reads:

> An improved shoe sole for increasing the resistance to slip on a contact surface, the sole comprising a bottom surface and defining a perimeter bounding a forefoot portion corresponding to the forefoot of the shoe and a heel portion corresponding to the heel of the shoe, [*1197] wherein the sole extends generally along a fore-aft axis running from said heel portion to said forefoot portion, the sole further comprising a substantially regular tiling array of projections projecting from said bottom surface, *said projections terminating in hexagonal shaped projected surfaces* spaced from said bottom surface in a direction for making contact with the contact surface, *said projections being oriented so that opposite edges of said projected surfaces face generally in the directions of said fore-aft axis*, said projected surfaces being substantially flat and parallel to the bottom surface to maximize the area of contact with [**3] the contact surface, said projections being spaced from one another to define substantially continuous channels therebetween for conducting liquid, *said channels being open over at least most of said perimeter*,

> *said forefoot portion and said heel portion of the sole.*

'198 application, at 7 (emphases added).

Three limitations of this claim are at issue, namely the limitations that: (A) the perimeter of the shoe is mostly open, (B) the projected surfaces, also called studs, are hexagonal in shape, and (C) the hexagonal shapes be oriented so that opposite edges of the hexagon "face generally in the directions of said fore-aft axis." *Id.* A figure from the '198 application is reproduced below, with non-substantive modifications for simplicity of presentation.

Prior art related to the '198 application includes *U.S. Patent No. 3,793,750* ("Bowerman"), *U.S. Design Patent No. 281462* ("Pope"), *U.S. Design Patent No. 263645* ("Mastrantuone"), and United Kingdom Patent No. 513,375 ("Davies"). Figures from these patents are reproduced below.

As can be seen in the figures, the orientation of the projected surfaces in these figures is different. In this opinion, we will refer [**4] to the orientation in the '198 application, Bowerman, and Pope as a "facing" orientation because the front edge of each hexagonal projected surface faces forward and the orientation in Mastrantuone and Davies as a "pointing" orientation.

GET DRAWING SHEET 1 OF 1

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GET DRAWING SHEET 1 OF 3

[*1198] The examiner rejected the '198 application, *inter alia*, on obviousness grounds by considering Pope in light of Bowerman and Davies, and appellants appealed this rejection to the Board. In its decision, the Board reversed the examiner's ground for rejection, supplied an alternative ground for rejection, and remanded. After [*1199] the Board entered its decision, appellants filed a request for rehearing. The panel held this motion for rehearing in abeyance while the examiner considered the application on remand. After reopening prosecution, the examiner rejected the '198 application for reasons identical to those offered by the Board in its first decision.

Appellants again appealed the examiner's rejection. In its decision, the Board "vacated the rejection of claim 1 set forth in the earlier decision in favor of the identical rejection later entered by the examiner." *Ex parte Fulton, 2003 Pat. App. LEXIS 88, No. 2003-0536, slip op. at 4 (Bd.* [**5] *Pat. App. & Int. Sept. 11, 2003).* The Board vacated the rejection in order to alleviate the confusion caused by the appellant in concurrently pursuing a request for a rehearing of the Board's first decision and a new appeal from the final rejection of the '198 application after remand. The Board credited the arguments in both actions. The Board then proceeded to affirm the rejection but under a different line of reasoning. The Board stated:

> In the present case, the combined teachings of Bowerman and Pope would have suggested the shoe sole recited in claim 1 to a person having ordinary skill in the art. As indicated above, Bowerman's shoe sole responds to all of the limitations in the claim except for those relating to the hexagonal shaped projected surfaces. While not specifically mentioning hexagonal shaped projected surfaces, Bowerman clearly suggests that cylindrical polygon shaped studs or projections other than those expressly described (square, rectangular or triangular) may be employed to provide sharp edges which bite into artificial turf for good traction. Pope establishes that shoe soles having studs embodying projected surfaces hexagonally shaped and oriented as recited [**6] in claim 1 are conventional. Given these disclosures, a person having ordinary skill in the art would have readily appreciated Pope's known hexagonal shaped projecting surfaces as being particularly well suited *for implementing* Bowerman's desire for projections having a plurality of sharp edges adapted to bite into artificial turf to obtain good traction. This appreciation would have furnished the artisan with ample suggestion or motivation to combine Bowerman and Pope in the manner proposed so as to arrive at the subject matter recited in claim 1.

*2003 Pat. App. LEXIS 88, *6-7.* After appellants' request for a rehearing was denied, they appealed to this court, which has jurisdiction under *28 U.S.C. § 1295(a)(4)(A).*

Discussion

I.

[HN1] "A patent may not be obtained ... if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains." *35 U.S.C. § 103(a).*

[HN2] Obviousness is a "question of law based on underlying findings of fact." *In re Gartside, 203 F.3d 1305, 1316 (Fed. Cir. 2000).* [**7] The Board's factual findings are upheld unless they are unsupported by substantial evidence. *Id.* Substantial evidence is "such relevant evidence as a reasonable mind might accept as adequate to support a conclusion." *Consol. Edison Co. v. NLRB, 305 U.S. 197, 229-30, 83 L. Ed. 126, 59 S. Ct. 206 (1938).* What the prior art teaches, whether it teaches away from the claimed invention, and whether it motivates a combination of teachings from different references are questions of [*1200] fact. *Id.*; *In re Berg, 320 F.3d 1310, 1312 (Fed. Cir. 2003).* Other factual findings related to obviousness may include "(1) the scope and content of the prior art; (2) the level of ordinary skill in the prior art; (3) the differences between the claimed invention and the prior art; and (4) objective evidence of nonobviousness." *In re Dembiczak, 175 F.3d 994, 998 (Fed. Cir. 1999), abrogated on other grounds in In re Gartside, 203 F.3d 1305 (Fed. Cir. 2000)* (abrogating the holding in *In re Dembiczak* that the Board's findings of fact are reviewed for clear error); *see also Graham v. John Deere Co., 383 U.S. 1, 17-18, 15 L. Ed. 2d 545, 86 S. Ct. 684 (1966).* [**8]

[HN3] "When a rejection depends on a combination of prior art references, there must be some teaching, suggestion, or motivation to combine the references." *In re Rouffet, 149 F.3d 1350, 1355 (Fed. Cir. 1998).* Stated another way, the prior art as a whole must "suggest the desirability" of the combination. *In re Beattie, 974 F.2d 1309, 1311 (Fed. Cir. 1992)* (internal quotation omitted); *Winner Int'l Royalty Corp. v. Wang, 202 F.3d 1340 (Fed. Cir. 2000)* ("Trade-offs often concern what is *feasible*, not what is, on balance, *desirable*. Motivation to combine requires the latter." (emphasis added)). The source of the teaching, suggestion, or motivation may be "the nature of the problem," "the teachings of the pertinent references," or "the ordinary knowledge of those skilled in the art." *In re Rouffet, 149 F.3d at 1355.*

II.

As quoted above, the Board found that the prior art as a whole suggested or motivated a combination of the open perimeter and orientation of Bowerman with the hexagonal surface and orientation of Pope. Appellants raise a number of arguments as to why this finding is not supported by substantial [**9] evidence.

Appellants first argue that the Board's finding of a motivation to combine lacks substantial evidence because the Board failed to demonstrate that the characteristics disclosed in Pope, hexagonal surfaces in a facing orientation, are preferred over other alternatives disclosed in the prior art. This argument fails because our [HN4] case law does not require that a particular combination must be the preferred, or the most desirable, combination described in the prior art in order to provide motivation for the current invention. "The question is whether there is something in the prior art as a whole to suggest the *desirability*, and thus the obviousness, of making the combination," not whether there is something in the prior art as a whole to suggest that the combination is the *most desirable* combination available. *See In re Beattie, 974 F.2d at 1311* (internal quotation omitted; emphasis added). A case on point is *In re Gurley, 27 F.3d 551, 552-53 (Fed. Cir. 1994)*, in which we upheld the Board's decision to reject, on obviousness grounds, the claims of a patent application directed to one of two alternative resins disclosed in a prior art [**10] reference, even though the reference described the resin claimed by Gurley as "inferior." Far from requiring that a disclosed combination be preferred in the prior art in order to be motivating, this court has held that "[a] known or obvious composition does not become patentable simply because it has been described as somewhat inferior to some other product for the same use" and the reference "teaches that epoxy is usable and has been used for Gurley's purpose." *Id.* Thus, a finding that the prior art as a whole suggests the desirability of a particular combination need not be supported by a finding that the prior art suggests that the combination claimed by the patent applicant is the preferred, or most desirable, combination.

[*1201] In this case, the Board found that "Bowerman clearly suggests that cylindrical polygon shaped studs or projections other than those expressly described (square, rectangular, or triangular) may be employed to provide sharp edges which bite into artificial turf for good traction." *Ex parte Fulton, 2003 Pat. App. LEXIS 88 at *7.* Bowerman thus provides a motivation to combine its teachings with other prior art references that disclose cylindrical polygon shapes other [**11] than squares, triangles, and rectangles. The Board also found that Pope discloses a shoe sole with hexagonal surfaces, which is a cylindrical polygon-shaped surface, and a facing orientation. Finally, the Board found that no other prior art references taught away from the combination of Bowerman and Pope that it adopted. These secondary findings are sufficient to support a primary finding that the prior art as a whole suggests the desirability of the combination of Bowerman and Pope described by the Board.

Appellants disagree with the Board's finding that no prior art references taught away from the combination of Bowerman and Pope adopted by the Board. Appellants quote language from *In re Gurley* that "[a] reference may be said to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference, or would be

led in a direction divergent from the path that was taken by the applicant." *27 F.3d at 553*. Appellants argue that "the prior art disclosed alternatives to each of the claimed elements A [the perimeter], B [the shape of the surface], and C [the orientation of the surface]. Choosing [**12] one alternative necessarily means rejecting the other, i.e., following a path that is 'in a divergent direction from the path taken by the'" applicant. This interpretation of our case law fails. The prior art's mere disclosure of more than one alternative does not constitute a teaching away from any of these alternatives because such disclosure does not criticize, discredit, or otherwise discourage the solution claimed in the '198 application. Indeed, in the case cited by appellants, *In re Gurley*, we held that the invention claimed in the patent application was unpatentable based primarily on a prior art reference that disclosed two alternatives, one of which was the claimed alternative. Accordingly, mere disclosure of alternative designs does not teach away.

Here, the design patents in the prior art disclose a number of alternative shoe sole designs but do not teach that hexagonal projections in a facing orientation are undesirable and, therefore, do not teach away. Furthermore, Davies communicates in its specification that its claimed invention, which includes hexagonal surfaces in a pointing orientation, has "a non-skid characteristic effective in all directions relative to [**13] its use." U.K. Patent No. 513,375 (accepted Oct. 11, 1939) at 2, ll. 19-20. But Davies does not teach that hexagons in a facing orientation would be ineffective. Accordingly, we find unpersuasive appellants' arguments that the prior art teaches away from hexagonal surfaces in a facing orientation.

Appellants next contend that the Board's finding lacks substantial evidence because it does not show a teaching in the prior art directed to the importance of aligning the cylindrical polygonal studs in a facing orientation. In their patent application, appellants assert that "this general orientation [a facing orientation] of the surfaces 36 has been found *optimal* for slip resistance in the sole of a shoe, in which there is a predetermined, usual or ordinary direction of travel." (Emphasis added.) Appellants' argument is unpersuasive from a legal standpoint because it again relies on the mistaken premise that the [*1202] prior art must teach that a particular combination is preferred, or "optimal," for the combination to be obvious. Furthermore, as we emphasized in *In re Beattie*, [HN5] "as long as some motivation or suggestion to combine the references is provided by the prior art taken as [**14] a whole, the law does not require that the references be combined for the reasons contemplated by the inventor." *974 F.2d at 1312*. Accordingly, this argument is unpersuasive because the Board need not have found the combination of Bowerman and Pope to be desirable for the reason stated in the '198 application.

This argument also fails on the facts of this case because the Board's findings are sufficiently broad to encompass appellants' idea of using a facing orientation because the predominant direction of travel is forward. The Board's finding that other cylindrical polygon shapes "may be employed to provide sharp edges which bite into artificial turf for good traction" suggests the importance of orientation because "bite" comes primarily from the front and back edges of the contact surface of a multi-sided stud being oriented so that the front edge faces the direction of travel and the back edge is directly opposite, as disclosed in Bowerman. *See* Bowerman, col. 2, ll. 55-60, figs. 2, 4. Indeed, in a discussion of "bite," Bowerman refers to Figures 2 and 4 of its specification, which depict a facing orientation. *Id.* Bite may also arise from the other edges of [**15] the contact surface, as well as edges formed by the intersection of the sides of the stud.

The Board also found that "a person having ordinary skill in the art would have readily appreciated Pope's known hexagonal shaped projecting surfaces as being particularly well suited for implementing Bowerman's desire for projections having a plurality of sharp edges adapted to bite into artificial turf to obtain good traction." *Ex parte Fulton, 2003 Pat. App. LEXIS 88 at *7*. Reasons why a hexagonal surface would be well-suited for obtaining good traction include the fact that the greater number of edges in a hexagon over a square provide bite in more directions. Further, although the Board's finding could perhaps have been clearer, it encompasses appellants' claim that a facing orientation is desirable because it provides bite in the forward direction. The Board's finding states that a person of ordinary skill of the art would have recognized that hexagonal surfaces as in Pope are "particularly well suited" to provide bite. *Id.* By referring to Pope, which has a facing orientation, rather than patents in the examination record that disclosed a pointing orientation, the Board's finding recognizes [**16] the importance of a facing orientation and, therefore, also the importance of providing "bite" in the forward direction.

Appellants finally contend that the Board did not properly weigh the prior art as required by *In re Young, 927 F.2d 588 (Fed. Cir. 1991)*, and did not provide sufficient reasoning for its rejection of these references as required by *In re Lee, 277 F.3d 1338 (Fed. Cir. 2002)*. Although the Board's analysis is short, the Board's decision is not so lacking in comparative reasoning that it fails under *In re Young* or *In re Lee*. The Board clearly considered the prior art cited by appellants and provided a factual basis upon which we can affirm its decision.

III.

The Board sustained the examiner's rejection of the dependent claims of the '198 application because "appellants have not challenged such with any reasonable specificity, thereby allowing these claims to stand or fall with parent claim 1." *Ex parte Fulton, 2003 Pat. App. LEXIS 88 at* *9. In its briefing before this court, appellants have also not raised [*1203] any arguments related solely to the dependent claims. Accordingly, because we affirm the Board's decision as to claim 1 of the [**17] '198 application, we also affirm the Board's decision as to the dependent claims.

Conclusion

In sum, the Board found that the prior art as a whole suggested or motivated a combination of the open perimeter of Bowerman with the hexagonal surface and facing orientation of Pope. Because this finding was supported by substantial evidence, we affirm the Board's rejection of the claims of the '198 application.

AFFIRMED

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Reference No. 17

LEXSEE 492 F.3D 1350

TAKEDA CHEMICAL INDUSTRIES, LTD. and TAKEDA PHARMACEUTICALS NORTH AMERICA, INC., Plaintiffs-Appellees, v. ALPHAPHARM PTY., LTD. and GENPHARM, INC., Defendants-Appellants.

06-1329

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

492 F.3d 1350; 2007 U.S. App. LEXIS 15349; 83 U.S.P.Q.2D (BNA) 1169

June 28, 2007, Decided

SUBSEQUENT HISTORY: Later proceeding at *Takeda Chem. Indus. v. Ranbaxy Labs., Ltd., 2007 U.S. App. LEXIS 15883 (Fed. Cir., June 28, 2007)*
US Supreme Court certiorari denied by Alphapharm Pty v. Takeda Chem. Indus., 2008 U.S. LEXIS 3015 (U.S., Mar. 31, 2008)

PRIOR HISTORY: [**1]
Appealed from: United States District Court for the Southern District of New York Judge Denise Cote.
Takeda Chem. Indus. v. Mylan Labs., Inc., 417 F. Supp. 2d 341, 2006 U.S. Dist. LEXIS 6710 (S.D.N.Y., 2006)

DISPOSITION: AFFIRMED.

CASE SUMMARY:

PROCEDURAL POSTURE: Defendant, the manufacturer of a generic version of pioglitazone, a compound successful in anti-diabetic treatment, appealed from a holding of the United States District Court for the Southern District of New York that the generic manufacturer failed to prove by clear and convincing evidence that the patent claims asserted by plaintiff patent holder were invalid as obvious under *35 U.S.C.S. § 103*, at the time the invention was made.

OVERVIEW: The generic manufacturer filed an abbreviated new drug application seeking U.S. Food and Drug Administration approval to market its generic product under *21 U.S.C.S. § 355(j) et seq.* The district court rejected the obviousness argument, because the closest prior art compound exhibited negative properties that would have directed one of ordinary skill in the art away from the compound that was eventually patented as the lead compound for anti-diabetic treatment. The most similarly structured compounds exhibited negative side effects, while other compounds were more promising at the time of invention. The court of appeals concluded that the district court did not err in determining that the claimed compounds would not have been obvious in light of the prior art, and that the patent had not been shown to be invalid. The trial also did not err in holding that the generic manufacturer failed to establish a prima facie case of obviousness. Because the district court's conclusions were not clearly erroneous and were supported by the record evidence, there was no basis to disturb them.

OUTCOME: The judgment of the district court was affirmed.

LexisNexis(R) Headnotes

Patent Law > Nonobviousness > General Overview
[HN1] An invention is not patentable, inter alia, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art. *35 U.S.C.S. § 103(a)*.

Patent Law > Infringement Actions > Defenses > Patent Invalidity > Validity Presumption
[HN2] Because a patent is presumed to be valid under *35 U.S.C.S. § 282*, the evidentiary burden to show facts supporting a conclusion of invalidity, which rests on the accused infringer, is one of clear and convincing evidence. Whether an invention would have been obvious under *35 U.S.C.S. § 103* is a question of law, reviewed de novo, based upon underlying factual questions which are reviewed for clear error following a bench trial.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN3] The factors that control an obviousness inquiry are: (1) the scope and content of the prior art; (2) the differences between the prior art and the claims; (3) the level of ordinary skill in the pertinent art; and (4) objective evidence of nonobviousness.

Patent Law > Nonobviousness > Elements & Tests > Prior Art
[HN4] Structural similarity between claimed and prior art subject matter, which is proven by combining references or otherwise where the prior art gives reason or motivation to make the claimed compositions, creates a prima facie case of obviousness. In addition to structural similarity between the compounds, a prima facie case of obviousness also requires a showing of adequate support in the prior art for the change in structure.

Patent Law > Nonobviousness > Evidence & Procedure > Prima Facie Obviousness
[HN5] Normally a prima facie case of obviousness is based upon structural similarity, that is, an established structural relationship between a prior art compound and the claimed compound. That is so because close or established structural relationships may provide the requisite motivation or suggestion to modify known compounds to obtain new compounds.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN6] A known compound may suggest its homolog, analog, or isomer because such compounds often have similar properties, and therefore chemists of ordinary skill would ordinarily contemplate making them to try to obtain compounds with improved properties. To find a prima facie case of unpatentability in such instances, a showing that the prior art would have suggested making the specific molecular modifications necessary to achieve the claimed invention was also required.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN7] Courts follow the standard statutory formulation under *35 U.S.C.S. § 103*, which is whether the claimed subject matter would have been obvious at the time the invention was made.

Patent Law > Nonobviousness > Elements & Tests > Predictability
[HN8] When there is a design need or market pressure to solve a problem, and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp.

Patent Law > Nonobviousness > Evidence & Procedure > Presumptions & Proof
[HN9] One who claims a compound, per se, which is structurally similar to a prior art compound, must rebut the presumed expectation that the structurally similar compounds have similar properties.

Patent Law > Nonobviousness > Evidence & Procedure > Prima Facie Obviousness
[HN10] Generalization should be avoided insofar as specific chemical structures are alleged to be prima facie obvious one from the other.

Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
[HN11] A person of ordinary skill is a hypothetical person who is presumed to be aware of all the pertinent prior art.

COUNSEL: David G. Conlin, Edwards Angell Palmer & Dodge LLP, of Boston, Massachusetts, argued for plaintiffs-appellees. With him on the brief were Barbara L. Moore, Kathleen B. Carr, and Adam P. Samansky; and Anthony J. Viola, of New York, New York. Of counsel on the brief was Mark Chao, Takeda Pharmaceuticals North America, Inc., of Lincolnshire, Illinois.

Kevin F. Murphy, Frommer Lawrence & Haug LLP, of New York, New York, argued for defendants-appellants. With him on the brief were Edgar H. Haug and Jeffrey A. Hovden.

JUDGES: Before LOURIE, BRYSON, and DYK, Circuit Judges. Opinion for the court filed by Circuit Judge LOURIE. Concurring opinion filed by Circuit Judge DYK. DYK, Circuit Judge, concurring.

OPINION BY: LOURIE

OPINION

[*1352] LOURIE, *Circuit Judge*.

Alphapharm Pty., Ltd. and Genpharm, Inc. (collectively "Alphapharm") appeal from the decision of the

United States District Court for the Southern District of New York, following a bench trial, that *U.S. Patent 4,687,777* was not shown to be invalid under *35 U.S.C. § 103*. *Takeda Chem. Indus., Ltd. v. Mylan Labs., 417 F. Supp. 2d 341 (S.D.N.Y. 2006)*. Because we conclude [**2] that the district court did not err in determining that the claimed compounds would not have been obvious in light of the prior art, and hence that the patent has not been shown to be invalid, we affirm.

BACKGROUND

Diabetes is a disease that is characterized by the body's inability to regulate blood sugar. It is generally caused by inadequate levels of insulin--a hormone produced in the pancreas. Insulin allows blood sugar or glucose, which is derived from food, to enter into the body's cells and be converted into energy. There are two types of diabetes, known as Type 1 and Type 2. In Type 1 diabetes, the pancreas fails to produce insulin, and individuals suffering from this type of diabetes must regularly receive insulin from an external source. In contrast, Type 2 diabetic individuals produce insulin. However, their bodies are unable to effectively use the insulin that is produced. This is also referred to as insulin resistance. As a result, glucose is unable to enter the cells, thereby depriving the body of its main source of energy. Type 2 diabetes is the most common form of diabetes--affecting over 90% of diabetic individuals.

In the 1990s, a class of drugs known as thiazolidinediones [**3] ("TZDs") was introduced on the market as a treatment for Type 2 diabetes. Takeda Chemical Industries, Ltd., and Takeda Pharmaceuticals North America, Inc. (collectively "Takeda") first invented certain TZDs in the 1970s. Takeda's research revealed that TZDs acted as insulin sensitizers, *i.e.*, compounds that ameliorate insulin resistance. Although the function of TZDs was not completely understood, TZDs appeared to lower blood glucose levels by binding to a molecule in the nucleus of the cell known as PPAR-gamma, which activates insulin receptors and stimulates the production of glucose transporters. *Takeda, 417 F. Supp. 2d at 348-49*. The transporters then travel to the cellular surface and enable glucose to enter the cell from the bloodstream. *Id.*

Takeda developed the drug ACTOS (R), which is used to control blood sugar in patients who suffer from Type 2 diabetes. ACTOS (R) has enjoyed substantial commercial success since its launch in 1999. By [*1353] 2003, it held 47% of the TZD market, and gross sales for that year exceeded $ 1.7 billion. *Id. at 386*. The active ingredient in ACTOS (R) is the TZD compound pioglitazone, a compound claimed in the patent in suit.

Takeda owns *U.S. Patent 4,687,777* [**4] (the "*'777 patent*") entitled "Thiazolidinedione Derivatives, Useful As Antidiabetic Agents." The patent is directed to "compounds which can be practically used as antidiabetic agents having a broad safety margin between pharmacological effect and toxicity or unfavorable side reactions." *'777 patent* col.1 ll.34-37. The asserted claims are claims 1, 2, and 5. Claim 1 claims a genus of compounds. Claim 5 claims pharmaceutical compositions containing that genus of compounds. Those claims read as follows:

> 1. A compound of the formula:
>
> [SEE DIAGRAM IN ORIGINAL]
>
> or a pharmacologically acceptable salt thereof.
>
> 5. An antidiabetic composition which consists essentially of a compound of the formula:
>
> [SEE DIAGRAM IN ORIGINAL]
>
> or a pharmacologically acceptable salt thereof, in association with a pharmacologically acceptable carrier, excipient or diluent.

Id., claims 1 & 5.

For purposes of this appeal, the critical portion of the compound structure is the left moiety of the molecule, namely, the ethyl-substituted pyridyl ring. [1] That chemical structure, which has an ethyl substituent (C[2]H[5]) pictorially drawn to the center of the pyridyl ring, indicates that the structure covers four possible compounds, [**5] *viz.*, compounds with an ethyl substituent located at the four available positions on the pyridyl ring. *Takeda, 417 F. Supp. 2d at 360*. The formula includes the 3-ethyl compound, 4-ethyl compound, 5-ethyl compound (pioglitazone), and 6-ethyl compound.

[*1354] Claim 2 of the *'777 patent* covers the single compound pioglitazone. That claim, which depends from claim 1, reads:

> 2. A compound as claimed in claim 1, wherein the compound is 5-4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl-2,4-thiazolidinedione.

'777 patent, claim 2. Pioglitazone is referred to as the 5-ethyl compound because the ethyl substituent is attached to the 5-position on the pyridyl ring. That portion of the compound is depicted as:

[SEE DIAGRAM IN ORIGINAL]

1 Pyridine is a "six-membered carbon-containing ring with one carbon replaced by a nitrogen." *Takeda, 417 F. Supp. 2d at 351.*

Alphapharm, a generic drug manufacturer, filed an Abbreviated New Drug Application ("ANDA") pursuant to the Hatch-Waxman Act seeking U.S. Food and Drug Administration ("FDA") approval under *21 U.S.C. § 355(j) et seq.* to manufacture and sell a generic version of pioglitazone. Alphapharm filed a Paragraph IV certification with its ANDA pursuant to § 505(j)(2)(B)(ii), [**6] asserting that the *'777 patent* is invalid as obvious under *35 U.S.C. § 103*. In response, Takeda sued Alphapharm, along with three other generic drug manufacturers who also sought FDA approval to market generic pioglitazone, alleging that the defendants have infringed or will infringe the *'777 patent*.

On January 17, 2006, the district court commenced a bench trial solely on the issues of validity and enforceability of the *'777 patent*. Alphapharm advanced its invalidity argument, asserting that the claimed compounds would have been obvious at the time of the alleged invention. Alphapharm's obviousness contention rested entirely on a prior art TZD compound that is referenced in Table 1 of the *'777 patent* as compound b. The left moiety of compound b consists of a pyridyl ring with a methyl (CH[3]) group attached to the 6-position of the ring. That portion of its chemical structure is illustrated as follows:

[SEE DIAGRAM IN ORIGINAL]

Alphapharm asserted that the claimed compounds would have been obvious over compound b.

The district court found that Alphapharm failed to prove by clear and convincing evidence that the asserted claims were invalid as obvious under *35 U.S.C. § 103*. The court first [**7] concluded that there was no motivation in the prior art to select compound b as the lead compound for antidiabetic research, and that the prior art taught away from its use. As such, the court concluded that Alphapharm failed to make a prima facie case of obviousness. The court continued its analysis and found that even if Alphapharm succeeded in making a prima facie showing, Takeda would still prevail because any prima facie case of obviousness was rebutted by the unexpected results of pioglitazone's nontoxicity. The court then rendered judgment in favor of Takeda. The district court also held that the *'777 patent* had not been procured though inequitable conduct. That decision has been separately appealed and has been affirmed in a decision issued today.

Alphapharm timely appealed. We have jurisdiction pursuant to *28 U.S.C. § 1295(a)(1)*.

DISCUSSION

A. Standard of Review

In this appeal, we are presented with one issue, namely, whether the asserted [*1355] claims of the *'777 patent* would have been obvious under *35 U.S.C. § 103* at the time the invention was made. [HN1] An invention is not patentable, *inter alia*, "if the differences between the subject matter sought to be patented and the prior art are [**8] such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art." *35 U.S.C. § 103(a)*. [HN2] Because a patent is presumed to be valid, *35 U.S.C. § 282*, the evidentiary burden to show facts supporting a conclusion of invalidity, which rests on the accused infringer, is one of clear and convincing evidence. *AK Steel Corp. v. Sollac & Ugine, 344 F.3d 1234, 1238-39 (Fed. Cir. 2003)*. Whether an invention would have been obvious under *35 U.S.C. § 103* is a "question of law, reviewed de novo, based upon underlying factual questions which are reviewed for clear error following a bench trial." *Alza Corp. v. Mylan Labs., Inc., 464 F.3d 1286, 1289 (Fed. Cir. 2006)*.

B. Obviousness

Alphapharm raises three main arguments in support of its contention that the claims would have been obvious. First, Alphapharm asserts that the district court misapplied the law, particularly the law governing obviousness in the context of structurally similar chemical compounds. According to Alphapharm, the record established that compound b was the most effective antidiabetic compound in the prior art, and thus the court erred by failing to apply [**9] a presumption that one of ordinary skill in the art would have been motivated to make the claimed compounds. Alphapharm asserts that such a conclusion is mandated by our case law, including our en banc decision in *In re Dillon, 919 F.2d 688 (Fed. Cir. 1990)*. Second, Alphapharm argues that the court erred in determining the scope and content of the prior art, in particular, whether to include the prosecution history of the prior *'779 patent*. Lastly, Alphapharm assigns error to numerous legal and factual determinations and certain evidentiary rulings that the court made during the course of the trial.

Takeda responds that the district court correctly determined that Alphapharm failed to prove by clear and convincing evidence that the asserted claims are invalid as obvious. Takeda contends that there was overwhelming evidence presented at trial to support the court's conclusion that no motivation existed in the prior art for one of ordinary skill in the art to select compound b as a lead compound, and even if there was, that the unexpected results of pioglitazone's improved toxicity would have rebutted any prima facie showing of obviousness. Ta-

keda further argues that all of Alphapharm's [**10] remaining challenges to the district court's legal and factual rulings are simply without merit.

We agree with Takeda that the district court did not err in concluding that the asserted claims of the *'777 patent* would not have been obvious. The Supreme Court recently addressed the issue of obviousness in *KSR International Co. v. Teleflex Inc., 127 S. Ct. 1727, 167 L. Ed. 2d 705 (2007)*. The Court stated that[HN3] the *Graham v. John Deere Co. of Kansas City, 383 U.S. 1, 86 S. Ct. 684, 15 L. Ed. 2d 545 (1966)*, factors still control an obviousness inquiry. Those factors are: 1) "the scope and content of the prior art"; 2) the "differences between the prior art and the claims"; 3) "the level of ordinary skill in the pertinent art"; and 4) objective evidence of nonobviousness. *KSR, 127 S. Ct. at 1734* (quoting *Graham, 383 U.S. at 17-18*).

In a thorough and well-reasoned opinion, albeit rendered before *KSR* was decided [*1356] by the Supreme Court, the district court made extensive findings of fact and conclusions of law as to the four *Graham* factors. Alphapharm's arguments challenge the court's determinations with respect to certain of these factors, which we now address.

1. Differences Between the Prior Art and the Claims

a. Selection of Compound b as Lead [**11] *Compound*

Alphapharm's first argument challenges the court's determination with regard to the "differences between the prior art and the claims." Alphapharm contends that the court erred as a matter of law in holding that the ethyl-substituted TZDs were nonobvious in light of the closest prior art compound, compound b, by misapplying the law relating to obviousness of chemical compounds.

We disagree. Our case law concerning prima facie obviousness of structurally similar compounds is well-established. We have held that [HN4] "structural similarity between claimed and prior art subject matter, proved by combining references or otherwise, where the prior art gives reason or motivation to make the claimed compositions, creates a prima facie case of obviousness." *Dillon, 919 F.2d at 692*. In addition to structural similarity between the compounds, a prima facie case of obviousness also requires a showing of "adequate support in the prior art" for the change in structure. *In re Grabiak, 769 F.2d 729, 731-32 (Fed. Cir. 1985)*.

We elaborated on this requirement in the case of *In re Deuel, 51 F.3d 1552, 1558 (Fed. Cir. 1995)*, where we stated that[HN5] "[n]ormally a prima facie case of obviousness is based [**12] upon structural similarity, *i.e.*, an established structural relationship between a prior art compound and the claimed compound." That is so because close or established "[s]tructural relationships may provide the requisite motivation or suggestion to modify known compounds to obtain new compounds." *Id.* [HN6] A known compound may suggest its homolog, analog, or isomer because such compounds "often have similar properties and therefore chemists of ordinary skill would ordinarily contemplate making them to try to obtain compounds with improved properties." *Id.* We clarified, however, that in order to find a prima facie case of unpatentability in such instances, a showing that the "prior art would have suggested making the specific molecular modifications necessary to achieve the claimed invention" was also required. *Id.* (citing *In re Jones, 958 F.2d 347 (Fed. Cir. 1992)*; *Dillon, 919 F.2d 688*; *Grabiak, 769 F.2d 729*; *In re Lalu, 747 F.2d 703 (Fed. Cir. 1984)*).

That test for prima facie obviousness for chemical compounds is consistent with the legal principles enunciated in *KSR*. [2] While the *KSR* Court rejected a rigid application of the teaching, suggestion, or motivation ("TSM") test in an obviousness [**13] inquiry, the Court acknowledged the importance of identifying "a reason that would have prompted a person of ordinary skill in the relevant field to combine [*1357] the elements in the way the claimed new invention does" in an obviousness determination. *KSR, 127 S. Ct. at 1731*. Moreover, the Court indicated that there is "no necessary inconsistency between the idea underlying the TSM test and the *Graham* analysis." *Id.* As long as the test is not applied as a "rigid and mandatory" formula, that test can provide "helpful insight" to an obviousness inquiry. *Id.* Thus, in cases involving new chemical compounds, it remains necessary to identify some reason that would have led a chemist to modify a known compound in a particular manner to establish prima facie obviousness of a new claimed compound.

2 We note that the Supreme Court in its *KSR* opinion referred to the issue as whether claimed subject matter "was" or "was not" obvious. Since *35 U.S.C. § 103* uses the language "would have been obvious," and the Supreme Court in *KSR* did consider the particular time at which obviousness is determined, we consider that[HN7] the Court did not in *KSR* reject the standard statutory formulation of the inquiry whether [**14] the claimed subject matter "would have been obvious at the time the invention was made." *35 U.S.C. § 103*. Hence, we will continue to use the statutory "would have been" language.

We agree with Takeda and the district court that Alphapharm failed to make that showing here. Alphapharm argues that the prior art would have led one of ordinary skill in the art to select compound b as a lead compound.

By "lead compound," we understand Alphapharm to refer to a compound in the prior art that would be most promising to modify in order to improve upon its antidiabetic activity and obtain a compound with better activity. [3] Upon selecting that compound for antidiabetic research, Alphapharm asserts that one of ordinary skill in the art would have made two obvious chemical changes: first, homologation, *i.e.*, replacing the methyl group with an ethyl group, which would have resulted in a 6-ethyl compound; and second, "ring-walking," or moving the ethyl substituent to another position on the ring, the 5-position, thereby leading to the discovery of pioglitazone. Thus, Alphapharm's obviousness argument clearly depends on a preliminary finding that one of ordinary skill in the art would have selected [**15] compound b as a lead compound.

> 3 The parties do not dispute that compound b was the closest prior art compound. Thus, the legal question is whether or not the claimed subject matter would have been obvious over that compound. We will, however, use Alphapharm's terminology of "lead compound" in this opinion, deciding the appeal as it has been argued.

The district court found, however, that one of ordinary skill in the art would not have selected compound b as the lead compound. In reaching its determination, the court first considered Takeda's *U.S. Patent 4,287,200* (the "*'200 patent*"), which was issued on September 1, 1981, and its prosecution history. The court found that the *'200 patent* "discloses hundreds of millions of TZD compounds." [4] *Takeda, 417 F. Supp. 2d at 378*. The patent specifically identified fifty-four compounds, including compound b, that were synthesized according to the procedures described in the patent, but did not disclose experimental data or test results for any of those compounds. The prosecution history, however, disclosed test results for nine specific compounds, including compound b. That information was provided to the examiner in response to a rejection in [**16] order to show that the claimed compounds of the *'200 patent* were superior to the known compounds that were disclosed in a cited reference. The court, however, found nothing in the *'200 patent*, or in its file history, to suggest to one of ordinary skill in the art that those nine compounds, out of the hundreds of millions of compounds covered by the patent application, were the best performing compounds as antidiabetics, and hence targets for modification to seek improved properties. *Id. at 375*.

> 4 Three divisional applications derive from the *'200 patent*. Those applications matured into *U.S. Patent 4,340,605*, *U.S. Patent 4,438,141*, and *U.S. Patent No. 4,444,779* (the "*'779 Patent*"). The *'779 patent* is of particular relevance in this appeal and is discussed below. *Takeda, 417 F. Supp. 2d at 378*.

[*1358] The court next considered an article that was published the following year in 1982 by T. Sodha et al. entitled "Studies on Antidiabetic Agents. II. Synthesis of 5-[4-(1-Methylcyclohexylmethoxy)-benzyl]thiazolidine-2,4-dione (ADD-3878) and Its Derivatives" ("Sodha II"). The Sodha II reference disclosed data relating to hypoglycemic activity and plasma triglyceride lowering activity for 101 TZD compounds. [**17] Those compounds did not include pioglitazone, but included compound b. Significantly, Sodha II identified three specific compounds that were deemed most favorable in terms of toxicity and activity. Notably, compound b was not identified as one of the three most favorable compounds. On the contrary, compound b, was singled out as causing "considerable increases in body weight and brown fat weight."

The court also considered Takeda's *'779 patent*. That patent covers a subset of compounds originally included in the *'200 patent* application, namely, TZD compounds "where the pyridyl or thiazolyl groups may be substituted." *Id. at 353*. The broadest claim of the *'779 patent* covers over one million compounds. *Id. at 378*. Compound b was specifically claimed in claim 4 of the patent. The court noted that a preliminary amendment in the prosecution history of the patent contained a statement that "the compounds in which these heterocyclic rings are substituted have become important, especially [compound b]." *Id.*

Based on the prior art as a whole, however, the court found that a person of ordinary skill in the art would not have selected compound b as a lead compound for antidiabetic treatment. Although [**18] the prosecution history of the *'779 patent* included the statement that characterized compound b as "especially important," the court found that any suggestion to select compound b was essentially negated by the disclosure of the Sodha II reference. The court reasoned that one of ordinary skill in the art would not have chosen compound b, notwithstanding the statement in the *'779 patent* prosecution history, "given the more exhaustive and reliable scientific analysis presented by Sodha II, which taught away from compound b, and the evidence from all of the TZD patents that Takeda filed contemporaneously with the '779 [p]atent showing that there were many promising, broad avenues for further research." *Id. at 380*.

The court found that the three compounds that the Sodha II reference identified as "most favorable" and "valuable for the treatment of maturity-onset diabetes," not compound b, would have served as the best "starting point for further investigation" to a person of ordinary skill in the art. *Id. at 376*. Because diabetes is a chronic

disease and thus would require long term treatment, the court reasoned that researchers would have been dissuaded from selecting a lead compound that [**19] exhibited negative effects, such as toxicity, or other adverse side effects, especially one that causes "considerable increases in body weight and brown fat weight." *Id. at 376-77*. Thus, the court determined that the prior art did not suggest to one of ordinary skill in the art that compound b would be the best candidate as the lead compound for antidiabetic research.

Admissions from Alphapharm witnesses further buttressed the court's conclusion. Dr. Rosenberg, head of Alphapharm's intellectual property department, testified as a 30(b)(6) witness on behalf of Alphapharm. In discussing Sodha II, Dr. Rosenberg admitted that there was nothing in [*1359] the article that would recommend that a person of ordinary skill in the art choose compound b over other compounds in the article that had the same efficacy rating. Dr. Rosenberg, acknowledging that compound b had the negative side effects of increased body weight and brown fat, also admitted that a compound with such side effects would "presumably not" be a suitable candidate compound for treatment of Type II diabetes. Alphapharm's expert, Dr. Mosberg, concurred in that view at his deposition when he admitted that a medicinal chemist would find [**20] such side effects "undesirable."

Moreover, another Alphapharm 30(b)(6) witness, Barry Spencer, testified at his deposition that in reviewing the prior art, one of ordinary skill in the art would have chosen three compounds in Sodha II as lead compounds for research, not solely compound b. In addition, Takeda's witness, Dr. Morton, testified that at the time Sodha II was published, it was known that obesity contributed to insulin resistance and Type 2 diabetes. Thus, one of ordinary skill in the art would have concluded that Sodha II taught away from pyridyl compounds because it associated adverse side effects with compound b.

We do not accept Alphapharm's assertion that *KSR*, as well as another case recently decided by this court, *Pfizer, Inc. v. Apotex, Inc., 480 F.3d 1348 (Fed. Cir. 2007)*, mandates reversal. Relying on *KSR*, Alphapharm argues that the claimed compounds would have been obvious because the prior art compound fell within "the objective reach of the claim," and the evidence demonstrated that using the techniques of homologation and ring-walking would have been "obvious to try." Additionally, Alphapharm argues that our holding in *Pfizer*, where we found obvious certain claims [**21] covering a particular acid-addition salt, directly supports its position.

We disagree. The *KSR* Court recognized that[HN8] "[w]hen there is a design need or market pressure to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp." *KSR, 127 S. Ct. at 1732*. In such circumstances, "the fact that a combination was obvious to try might show that it was obvious under *§ 103*." *Id.* That is not the case here. Rather than identify predictable solutions for antidiabetic treatment, the prior art disclosed a broad selection of compounds any one of which could have been selected as a lead compound for further investigation. Significantly, the closest prior art compound (compound b, the 6-methyl) exhibited negative properties that would have directed one of ordinary skill in the art away from that compound. Thus, this case fails to present the type of situation contemplated by the Court when it stated that an invention may be deemed obvious if it was "obvious to try." The evidence showed that it was not obvious to try.

Similarly, Alphapharm's reliance on *Pfizer* fares no better. [**22] In *Pfizer*, we held that certain claims covering the besylate salt of amlodipine would have been obvious. The prior art included a reference, referred to as the Berge reference, that disclosed a genus of pharmaceutically acceptable anions that could be used to form pharmaceutically acceptable acid addition salts, as well as other publications that disclosed the chemical characteristics of the besylate salt. *Pfizer, 480 F.3d at 1363*. Noting that our conclusion was based on the "particularized facts of this case," we found that the prior art provided [*1360] "ample motivation to narrow the genus of 53 pharmaceutically-acceptable anions disclosed by Berge to a few, including benzene sulphonate." *Id. at 1363, 1367*. Here, the court found nothing in the prior art to narrow the possibilities of a lead compound to compound b. In contrast, the court found that one of ordinary skill in the art would have chosen one of the many compounds disclosed in Sodha II, of which there were over ninety, that "did not disclose the existence of toxicity or side effects, and to engage in research to increase the efficacy and confirm the absence of toxicity of those compounds, rather than to choose as a starting point [**23] a compound with identified adverse effects." Thus, *Pfizer* does not control this case.

Based on the record before us, we conclude that the district court's fact-findings were not clearly erroneous and were supported by evidence in the record. Moreover, we reject the assertion that the court failed to correctly apply the law relating to prima facie obviousness of chemical compounds. Because Alphapharm's obviousness argument rested entirely on the court making a preliminary finding that the prior art would have led to the selection of compound b as the lead compound, and Alphapharm failed to prove that assertion, the court did not commit reversible error by failing to apply a presumption

of motivation. We thus conclude that the court did not err in holding that Alphapharm failed to establish a prima facie case of obviousness. *See Eli Lilly & Co. v. Zenith Goldline Pharms., 471 F.3d 1369 (Fed. Cir. 2006)* (affirming the district court's finding of nonobviousness upon concluding, in part, that the prior art compound would not have been chosen as a lead compound).

b. Choice of the Claimed Compounds

Even if Alphapharm had established that preliminary finding, and we have concluded that it did [**24] not, the record demonstrates that Alphapharm's obviousness argument fails on a second ground. The district court found nothing in the prior art to suggest making the specific molecular modifications to compound b that are necessary to achieve the claimed compounds. In reaching that conclusion, the court first found that the process of modifying lead compounds was not routine at the time of the invention. *Takeda, 417 F. Supp. 2d at 380.* Dr. Mosberg opined that the steps of homologation and ring-walking were "routine steps in the drug optimization process," but the court found that testimony unavailing in light of the contrary, more credible, testimony offered by Takeda's experts. *Id. at 381.* In addition, the court relied on Dr. Rosenberg's admission that a person of ordinary skill in the art would "look at a host of substituents, such as chlorides, halides and others, not just methyls" in modifying the pyridyl ring. *Id.*

Pioglitazone differs from compound b in two respects, and one would have to both homologate the methyl group of compound b and move the resulting ethyl group to the 5-position on the pyridyl ring in order to obtain pioglitazone. With regard to homologation, the court [**25] found nothing in the prior art to provide a reasonable expectation that adding a methyl group to compound b would reduce or eliminate its toxicity. Based on the test results of the numerous compounds disclosed in Sodha II, the court concluded that "homologation had no tendency to decrease unwanted side effects" and thus researchers would have been inclined "to focus research efforts elsewhere." *Id. at 383.* Indeed, several other compounds exhibited similar or better potency than compound b, and one compound in particular, compound 99, that had no identified problems differed significantly [*1361] from compound b in structure. *Id. at 376 n.51.* Moreover, Dr. Mosberg agreed with Takeda's expert, Dr. Danishefsky, that the biological activities of various substituents were "unpredictable" based on the disclosure of Sodha II. *Id. at 384-85.* The court also found nothing in the *'200* and *'779 patents* to suggest to one of ordinary skill in the art that homologation would bring about a reasonable expectation of success.

As for ring-walking, the court found that there was no reasonable expectation in the art that changing the positions of a substituent on a pyridyl ring would result in beneficial changes. [**26] Dr. Mosberg opined that the process of ring-walking was "known" to Takeda, but the court found that testimony inapt as it failed to support a reasonable expectation to one of ordinary skill in the art that performing that chemical change would cause a compound to be more efficacious or less toxic. *Id. at 382.* Moreover, Dr. Mosberg relied on the efficacy data of phenyl compounds in Sodha II, but the court found those data insufficient to show that the same effects would occur in pyridyl compounds.

Alphapharm relies on *In re Wilder, 563 F.2d 457 (CCPA 1977)*, for the proposition that differences in a chemical compound's properties, resulting from a small change made to the molecule, are reasonably expected to vary by degree and thus are insufficient to rebut a prima facie case of obviousness. In *Wilder*, our predecessor court affirmed the Board's holding that a claimed compound, which was discovered to be useful as a rubber antidegradant and was also shown to be nontoxic to human skin, would have been obvious in light of its homolog and isomer that were disclosed in the prior art. The evidence showed that the homolog was similarly nontoxic to the human skin, whereas the isomer was toxic. [**27] The court held that [HN9] "one who claims a compound, per se, which is structurally similar to a prior art compound must rebut the presumed expectation that the structurally similar compounds have similar properties." *Id. at 460.* While recognizing that the difference between the isomer's toxicity and the nontoxicity of the homolog and claimed compound "indicate[d] some degree of unpredictability," the court found that the appellant failed to "point out a single actual difference in properties between the claimed compound and the homologue," and thus failed to rebut the presumption. *Wilder, 563 F.2d at 460.*

We would note that since our *Wilder* decision, we have cautioned "that [HN10] generalization should be avoided insofar as specific chemical structures are alleged to be prima facie obvious one from the other," *Grabiak, 769 F.2d at 731.* In addition to this caution, the facts of the present case differ significantly from the facts of *Wilder*. Here, the court found that pioglitazone exhibited unexpectedly superior properties over the prior art compound b. *Takeda, 417 F. Supp. 2d at 385.* The court considered a report entitled "Preliminary Studies on Toxicological Effects of Ciglitazone-Related Compounds [**28] in the Rats" that was presented in February 1984 by Dr. Takeshi Fujita, then-Chief Scientist of Takeda's Biology Research Lab and co-inventor of the *'777 patent*. That report contained results of preliminary toxicity studies that involved selected compounds, including pioglitazone and compound b. Compound b was shown to be "toxic to the liver, heart and erythrocytes,

among other things," whereas pioglitazone was "comparatively potent" and "showed no statistically significant toxicity." *Id. at 356-57.* During the following months, Takeda performed [*1362] additional toxicity studies on fifty compounds that had been already synthesized and researched by Takeda, including pioglitazone. The compounds were tested for potency and toxicity. The results were presented in another report by Fujita entitled "Pharmacological and Toxicological Studies of Ciglitazone and Its Analogues." Pioglitazone was shown to be the only compound that exhibited no toxicity, although many of the other compounds were found to be more potent. *Id. at 358.*

Thus, the court found that there was no reasonable expectation that pioglitazone would possess the desirable property of nontoxicity, particularly in light of the toxicity [**29] of compound b. The court's characterization of pioglitazone's unexpected results is not clearly erroneous. As such, *Wilder* does not aid Alphapharm because, unlike the homolog and claimed compound in *Wilder* that shared similar properties, pioglitazone was shown to differ significantly from compound b, of which it was not a homolog, in terms of toxicity. Consequently, Takeda rebutted any presumed expectation that compound b and pioglitazone would share similar properties.

Alphapharm also points to a statement Takeda made during the prosecution of the *'779 patent* as evidence that there was a reasonable expectation that making changes to the pyridyl region of compound b would lead to "better toxicity than the prior art." During prosecution of the *'779 patent*, in response to an enablement rejection, Takeda stated that "there should be no reason in the instant case for the Examiner to doubt that the claimed compounds having the specified substituent would function as a hypolipidemic and hypoglycemic agent as specified in the instant disclosure." That statement, however, indicates only that changes to the left moiety of a lead compound would create compounds with the same properties as the [**30] compounds of the prior art; it does not represent that lower toxicity would result. And even if the statement did so represent, it does not refer to any specific substituent at any specific position of TZD's left moiety as particularly promising. As the court correctly noted, the compounds disclosed in the *'779 patent* included a variety of substituents, including lower alkyls, halogens, and hydroxyl groups, attached to a pyridyl or thiazolyl group. As discussed *supra*, the district court found that the claims encompassed over one million compounds. Thus, we disagree with Alphapharm that that statement provided a reasonable expectation to one of ordinary skill in the art that performing the specific steps of replacing the methyl group of the 6-methyl compound with an ethyl group, and moving that substituent to the 5-position of the ring, would have provided a broad safety margin, particularly in light of the district court's substantiated findings to the contrary.

We thus conclude that Alphapharm's challenges fail to identify grounds for reversible error. The court properly considered the teachings of the prior art and made credibility determinations regarding the witnesses at trial. [**31] We do not see any error in the district court's determination that one of ordinary skill in the art would not have been prompted to modify compound b, using the steps of homologation and ring-walking, to synthesize the claimed compounds. Because the court's conclusions are not clearly erroneous and are supported by the record evidence, we find no basis to disturb them.

The court properly concluded that Alphapharm did not make out a prima facie case of obviousness because Alphapharm [*1363] failed to adduce evidence that compound b would have been selected as the lead compound and, even if that preliminary showing had been made, it failed to show that there existed a reason, based on what was known at the time of the invention, to perform the chemical modifications necessary to achieve the claimed compounds.

In light of our conclusion that Alphapharm failed to prove that the claimed compounds would have been prima facie obvious, we need not consider any objective indicia of nonobviousness. [5]

> 5 The concurrence, while agreeing that the question of the "overbreadth" of claims 1 and 5 has been waived, states further that the 6-ethyl compound, which is within the scope of claims 1 and 5, has not been [**32] shown to possess unexpected results sufficient to overcome a prima facie case of obviousness, and hence claims 1 and 5 are likely invalid as obvious. Since waiver is sufficient to answer the point being raised, no further comment need be made concerning its substance.

2. Scope and Content of the Prior Art

Alphapharm also assigns error to the district court's determination regarding the scope and content of the prior art. Alphapharm asserts that the court excluded the prosecution history of the *'779 patent* from the scope of the prior art after wrongly concluding that it was not accessible to the public. Takeda responds that the court clearly considered the *'779 patent* prosecution history, which was admitted into evidence on the first day of testimony. Takeda urges that the court's consideration of the prosecution history is apparent based on its extensive analysis of the *'779 patent* and the file history that appears in the court's opinion.

We agree with Takeda that the district court did not err in its consideration of the scope of the prior art. As

discussed above, the court considered the prosecution history, and even expressly considered one of the key statements in the prosecution [**33] history upon which Alphapharm relies in support of its position that compound b would have been chosen as the lead compound. *Takeda, 417 F. Supp. 2d at 378.* In considering the prosecution history of the *'779 patent*, the court noted that Takeda filed a preliminary amendment on March 15, 1983, in which its prosecuting attorney stated that "the compounds in which these heterocyclic rings are substituted have become important, especially [the 6-methyl compound]." *Id.* The court rejected Alphapharm's assertion that that statement supported the conclusion that compound b would have been selected as a lead compound. Rather, the court found that viewing the prior art as a whole, the prior art showed "that Takeda was actively conducting research in many directions, and had not narrowed its focus to compound b." *Id. at 379.* Thus, while the district court may have incorrectly implied that prosecution histories are not accessible to the public, *see id.* at n.59, *see also Custom Accessories, Inc. v. Jeffrey-Allan Indus., 807 F.2d 955 (Fed. Cir. 1986)* ([HN11] "[t]he person of ordinary skill is a hypothetical person who is presumed to be aware of all the pertinent prior art"), the court nonetheless considered [**34] the prosecution history of the *'779 patent* in its obviousness analysis and accorded proper weight to the statements contained therein. Thus, any error committed by the court in this regard was harmless error.

We have considered Alphapharm's remaining arguments and find none that warrant reversal of the district court's decision.

[*1364] CONCLUSION

We affirm the district court's determination that claims 1, 2, and 5 of the *'777 patent* have not been shown to have been obvious and hence invalid.

AFFIRMED

CONCUR BY: DYK

CONCUR

DYK, *Circuit Judge*, concurring.

I join the opinion of the court insofar as it upholds the district court judgment based on a determination that a claim to pioglitazone (the 5-ethyl compound) would be non-obvious over the prior art. The problem is that only one of the three claims involved here--claim 2--is limited to pioglitazone. In my view, the breadth of the other two claims, claims 1 and 5 of *U.S. Patent No. 4,867,777 ("'777 patent")*--which are also referenced in the judgment--renders them likely invalid.

All of the compounds claimed in claims 1, 2 and 5 were included in generic claims in the prior art *U.S. Patent No. 4,287,200 ("'200 patent").* Unfortunately our law concerning when a species [**35] is patentable over a genus claimed in the prior art is less than clear. It is, of course, well established that a claim to a genus does not necessarily render invalid a later claim to a species within that genus. *See Eli Lilly & Co. v. Bd. of Regents of Univ. of Wash., 334 F.3d 1264, 1270 (Fed. Cir. 2003).* In my view a species should be patentable over a genus claimed in the prior art only if unexpected results have been established. Our case law recognizes the vital importance of a finding of unexpected results, both in this context and in the closely related context where a prior art patent discloses a numerical range and the patentee seeks to claim a subset of that range. *See Application of Petering, 301 F.2d 676, 683, 49 C.C.P.A. 993, 1962 Dec. Comm'r Pat. 232 (C.C.P.A. 1962)* (species found patentable when genus claimed in prior art because unexpected properties of the species were shown); *see also Pfizer, Inc. v. Apotex, Inc., 480 F.3d 1348, 1371 (Fed. Cir. 2007)* (relying on lack of unexpected results in determining that species claim was obvious in view of prior art genus claim); *In re Woodruff, 919 F.2d 1575, 1578 (Fed. Cir. 1990)* (when applicant claims a subset of a range disclosed in a prior art patent, the applicant [**36] must generally show that "the claimed range achieves unexpected results relative to the prior art range.").

While the 5-ethyl compound (pioglitazone) is within the scope of the *'200 patent*, there is clear evidence, as the majority correctly finds, of unexpected results regarding that compound, and therefore its validity is not in question on this ground. However, at oral argument the patentee admitted that the prior art *'200 patent* also generically covers the 6-ethyl compound, which is within the scope of claims 1 and 5 of the *'777 patent*, and admitted that there is no evidence of unexpected results for the 6-ethyl compound. Under such circumstances, I believe that the 6-ethyl is likely obvious, and consequently claims 1 and 5 are likely invalid for obviousness. However, the argument as to the overbreadth of claims 1 and 5 has been waived, because it was not raised in the opening brief. In any event, as a practical matter, the judgment finding that the appellants' filing of the ANDA for pioglitazone is an infringement and barring the making of pioglitazone is supported by the finding that claim 2 standing alone is not invalid and is infringed.

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LEXSEE 349 F.3D 1333

CFMT, INC. and CFM TECHNOLOGIES, INC., Plaintiffs-Appellants, v. YIELDUP INTERNATIONAL CORP., Defendant-Appellee.

01-1452

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

349 F.3d 1333; 2003 U.S. App. LEXIS 23072; 68 U.S.P.Q.2D (BNA) 1940

November 12, 2003, Decided

SUBSEQUENT HISTORY: Rehearing denied by *CFMT, Inc. v. Yieldup Int'l Corp., 2003 U.S. App. LEXIS 26684 (Fed. Cir., Dec. 9, 2003)*

PRIOR HISTORY: [**1] Appealed from: United States District Court for the District of Delaware. Judge Roderick R. McKelvie.
CFMT, Inc. v. Yieldup Int'l Corp., 144 F. Supp. 2d 305, 2001 U.S. Dist. LEXIS 7593 (D. Del., 2001)

CASE SUMMARY:

PROCEDURAL POSTURE: Plaintiff patent holders sued defendant competitors for patent infringement under *35 U.S.C.S. § 271*. The competitors denied infringing, claiming invalidity for nonenablement and unenforceability for inequitable conduct, and moved for summary judgment. The patent holder moved for summary judgment of enablement. After trial, the United States District Court for the District of Delaware held for the competitors. The patent holder appealed.

OVERVIEW: The patents covered a semiconductor wafer cleaning system. The district court based its nonenablement judgment on: (1) lack of utility or inoperability and (2) undue experimentation needed to carry out the invention. The court held that the district court erred in requiring that the patent disclosures enable a single embodiment. In essence, this set the *35 U.S.C.S. § 112* enablement bar too high. The lengthy experiments did not show nonenablement because the inventors undertook that work to satisfy commercial requirements, not to show enablement. While the record did not appear to present a genuine issue of material fact about whether a person of ordinary skill in the art could achieve any level of cleaning with the claimed invention without undue experimentation, this court remanded for reconsideration of that question. Moreover, the district court also clearly erred in finding that the applicants' statements were material misrepresentations. The statements were not inaccurate and the materiality of the undisclosed subject matter was low, so there was little basis for inferring intent. The district court clearly erred in finding in inequitable conduct in prosecuting the patents.

OUTCOME: The appellate court reversed and vacated the judgment and remanded for reconsideration.

LexisNexis(R) Headnotes

Civil Procedure > Summary Judgment > Appellate Review > Standards of Review
[HN1] A federal circuit court reviews without deference a district court's grant of summary judgment.

Civil Procedure > Summary Judgment > Burdens of Production & Proof > General Overview
Civil Procedure > Summary Judgment > Standards > General Overview
[HN2] A court considering summary judgment must view the evidence presented through the prism of the substantive evidentiary burden. The court must also draw all reasonable inferences in favor of the nonmovant.

Civil Procedure > Appeals > Standards of Review > De Novo Review
Patent Law > Infringement Actions > Claim Interpretation > General Overview
[HN3] An appellate court reviews patent claim construction without deference.

Civil Procedure > Appeals > Standards of Review > De Novo Review
Patent Law > Claims & Specifications > Enablement Requirement > Proof
[HN4] On a claim of patent infringement, enablement is a question of law with factual underpinnings; the appellate court reviews the ultimate legal conclusion without deference.

Civil Procedure > Appeals > Standards of Review > Clearly Erroneous Review
Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > Fact & Law Issues
Patent Law > Jurisdiction & Review > Standards of Review > Abuse of Discretion
[HN5] In the context of a patent infringement claim, the appellate court reviews a determination of inequitable conduct for abuse of discretion and reviews the underlying factual issues of materiality and intent for clear error.

Patent Law > Claims & Specifications > Enablement Requirement > Standards & Tests
Patent Law > Inequitable Conduct > General Overview
[HN6] Enablement does not require an inventor to meet lofty standards for success in the commercial marketplace. Title 35 does not require that a patent disclosure enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment absent a claim limitation to that effect. Title 35 requires only that the inventor enable one of skill in the art to make and use the full scope of the claimed invention. Thus, when an invention claims a general system to improve the cleaning process for semiconductor wafers, the disclosure enables that invention by showing improvements in the overall system. The enablement requirement is met if the description enables any mode of making and using the claimed invention. Of course, if a patent claimed a system that achieved cleanliness up to a specified numerical particle-free range, then enablement would require disclosure of a method that enables one of ordinary skill to achieve that range without undue experimentation. Thus, the level of disclosure necessary to satisfy *35 U.S.C.S. § 112* varies according to the scope of the claimed invention.

Patent Law > Claims & Specifications > Enablement Requirement > Scope
Patent Law > Claims & Specifications > Enablement Requirement > Standards & Tests
Patent Law > Inequitable Conduct > General Overview
[HN7] Patents are not production documents, and nothing in the patent law requires that a patentee must disclose data on how to mass-produce the invented product. The law requires that patents disclose inventions, not mass-production data, and that patents enable the practice of inventions, not the organization and operation of factories.

Patent Law > Claims & Specifications > Enablement Requirement > Standards & Tests
Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals
[HN8] On a patent infringement claim, the United States Court of Appeals for the Federal Circuit gauges enablement at the date of the filing, not in light of later developments.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Utility Requirement > Proof of Utility
[HN9] The United States Court of Appeals for the Federal Circuit has recognized the relationship between the enablement requirement of *35 U.S.C.S. § 112* and the utility requirement of *35 U.S.C.S. § 101*. If the claims in an application fail to meet the utility requirement because the invention is inoperative, they also fail to meet the enablement requirement because a person skilled in the art cannot practice the invention.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Utility Requirement > Proof of Utility
[HN10] An inoperable invention is not enabled.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Subject Matter > Products > Machines
Patent Law > Utility Requirement > Proof of Utility
[HN11] The inoperability standard for utility applies primarily to claims with impossible limitations. Moreover, where a patent discloses several alternative combinations of methods (as most systems claims will), the party asserting inoperability must show that all disclosed alternatives are inoperative or not enabled.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
[HN12] Improvement and selection inventions are ubiquitous in patent law; such developments do not alone cast doubt on enablement of the original invention. In general, few patented inventions are an immediate commercial success. Rather, most inventions require further de-

velopment to achieve commercial success. Thus, additional inventive work does not alone show nonenablement.

Patent Law > Claims & Specifications > Theory of Invention
Patent Law > Nonobviousness > Elements & Tests > Manner of Conception
Patent Law > Statutory Bars > Experimental Use > Elements
[HN13] Patent acquisition does not require any threshold level of effort or ingenuity. *35 U.S.C.S. § 103(a)*. Patentability shall not be negatived by the manner in which the invention was made. *35 U.S.C.S. § 103*. It is immaterial whether the invention resulted from long toil and experimentation or from a flash of genius. The path that leads an inventor to the invention is expressly made irrelevant to patentability by statute. Thus, an improvement patent alone is not conclusive evidence of undue experimentation.

Evidence > Procedural Considerations > Burdens of Proof > Clear & Convincing Proof
Patent Law > Inequitable Conduct > Burdens of Proof
Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > Elements
[HN14] Inequitable conduct requires proof that a patent applicant did not disclose material information to the Patent and Trademark Office with intent to deceive. More specifically, inequitable conduct includes affirmative misrepresentation of a material fact, failure to disclose material information, or submission of false material information, coupled with an intent to deceive. These elements must be shown with clear and convincing evidence. Under the pre-1992 standard for materiality standard, information is material if there is a substantial likelihood that a reasonable examiner would consider it important in deciding whether to allow the application to issue as a patent.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN15] Obviousness requires a suggestion of all limitations in a claim.

Patent Law > Inequitable Conduct > General Overview
Patent Law > Nonobviousness > Elements & Tests > General Overview
Patent Law > Nonobviousness > Evidence & Procedure > Prima Facie Obviousness
[HN16] A patent applicant cannot prove unexpected results with attorney argument and bare statements without objective evidentiary support. It is well settled that unexpected results must be established by factual evidence. Mere argument or conclusory statements do not suffice. During patent prosecution, an applicant may submit objective factual evidence to the Patent and Trademark Office in the form of patents, technical literature, and declarations under *37 C.F.R. § 1.132 (2003)* submitting expert testimony and, at times, test data.

Patent Law > Inequitable Conduct > Burdens of Proof
[HN17] A district court may infer intent to deceive the Patent and Trademark Office. However, even gross negligence does not alone suffice to establish intent. Instead, the involved conduct, viewed in light of all the evidence, including evidence indicative of good faith, must indicate sufficient culpability to require a finding of intent to deceive.

COUNSEL: Henrik D. Parker, Woodcock Washburn LLP, of Philadelphia, Pennsylvania, argued for plaintiffs-appellants. With him on the brief were Barbara L. Mullin, Richard B. LeBlanc, and David N. Farsiou. Of counsel on the brief was Fred T. Magaziner, Dechert LLP, of Philadelphia, Pennsylvania.

L. Gene Spears, Baker Botts L.L.P., of Houston, Texas, argued for defendant-appellee. With him on the brief was David G. Wille, Baker Botts, L.L.P., of Dallas, Texas.

JUDGES: Before RADER, Circuit Judge, FRIEDMAN, Senior Circuit Judge, and LINN, Circuit Judge.

OPINION BY: RADER

OPINION

[*1334] RADER, *Circuit Judge*.

On summary judgment, the United States District Court for the District of Delaware determined that CFMT, Inc.'s *U.S. Patent No. 4,778,532* (the *'532 patent*) and *U.S. Patent No. 4,917,123* (the *'123 patent*) are invalid, *CFMT, Inc. v. YieldUp Int'l Corp., 92 F. Supp. 2d 359 (D. Del. 2000)*, and, after a bench trial, unenforceable, *CFMT, Inc. v. YieldUp Int'l Corp., 144 F. Supp. 2d 305 (D. Del. 2001)*. Because the district court erred in applying both the enablement and [**2] inequitable conduct requirements, this court reverses-in-part, vacates-in-part, and remands.

[*1335] I.

The '532 and *'123 patents* cover a system for cleaning semiconductor wafers. The process for manufactur-

ing semiconductor wafers must keep them as free as possible from contamination to prevent defects in semiconductors. To keep the wafers clean, conventional processes sequentially immerse the wafers in various liquids in an open environment. This bathing procedure exposes the wafers to airborne contaminants and also exposes workers to hazardous chemicals.

The '532 and *'123 patents* claim improvements in these open cleaning systems. Specifically, the '532 and *'123 patents* claim a system that is closed to the outside environment and requires no human handling. Instead the wafers remain at all times in a closed container that sequentially introduces different chemicals to clean the wafers. Because the *'123 patent* is a divisional of the *'532 patent*, the two patents have identical disclosures. The parent *'532 patent* contains method claims only. Independent claims 1 and 55 are representative (emphases added):

> 1. An enclosed, full flow method for the *cleaning* of semiconductor wafers [**3] comprising positioning said wafers in a vessel, closing said vessel to the environment, and flowing process fluids sequentially and continuously past said wafers in said vessel, including the steps of
>
> (a) contacting said wafers with at least one cleaning fluid to remove contaminants from said wafers;
>
> (b) removing said cleaning fluid from said wafers with a rinsing fluid; and
>
> (c) removing said rinsing fluid from said wafers with a drying fluid;
>
> whereby the processing does not requirement [sic] movement or operator handling of said wafers between said steps; and maintaining the vessel containing said wafers hydraulically full during each process step.
>
> 55. An enclosed, full flow method for the *treatment* of semiconductor wafers comprising positioning said wafers in a vessel, closing said vessel to the environment, and flowing process fluids in sequential steps continuously past said wafers in said vessel, including the step of reacting the surface of said wafers with at least one chemical reagent, whereby the processing does not require movement or handling of said wafers between said steps and maintaining the vessel containing said wafers hydraulically full during each [**4] process step.

The divisional *'123 patent* contains corresponding apparatus claims. Independent claims 1 and 20 are representative (emphases added):

> 1. Apparatus for *wet processing* of semiconductor wafers comprising:
>
> (a) vessel means for supporting said wafers in a closed circulation process stream wherein process fluids may sequentially flow past said wafers, said vessel being hydraulically full with process fluid when said process fluids flow past said wafers;
>
> (b) means for supplying at least one cleaning fluid to said process stream for removing contaminants from said wafers, and means for withdrawing said cleaning fluid from said process stream;
>
> (c) means for supplying a rinsing fluid to said process stream for removing other fluids from said wafers, means for minimizing gas/liquid interfaces in said rinsing fluid and means for withdrawing said rinsing fluid from said process stream; and
>
> [*1336] (d) means for supplying a drying fluid to said process stream for removing other fluids from said wafers and means for withdrawing said drying fluid from said process stream.
>
> 20. Apparatus for *wet processing* of semiconductor wafers comprising:
>
> (a) vessel means for supporting [**5] said wafers in a closed circulation process stream wherein process fluids may sequentially flow past said wafers and
>
> (b) means for supplying at least one chemical reagent to said process stream for reacting with portions of said wafers, said process stream being positioned within said vessel means such that said vessel means is hydraulically full with process fluid.

The record in this case shows that the inventors installed for Texas Instruments (TI) a machine that per-

formed the claimed method. At first the apparatus did not meet this customer's standards for wafer cleanliness. The inventors adjusted the apparatus and experimented for months before meeting the customer's standards. In fact, the inventors obtained a third patent claiming the improvements in their initial apparatus.

CFMT and CFM Technologies, Inc.[1] (collectively CFMT) sued YieldUp International Corp. (YieldUp) for infringement of the '532 and *'123 patents*. In turn, YieldUp denied infringing and asserted that the patents were invalid as nonenabled and were unenforceable for inequitable conduct before the United States Patent and Trademark Office (PTO). YieldUp moved for summary judgment that the patents were invalid [**6] for lack of enablement. CFMT filed a cross-motion for summary judgment that the patents were enabled.

1 CFM Technologies, Inc. assigned the patents to holding company CFMT, Inc., which in turn granted CFM Technologies, Inc. an exclusive license.

YieldUp based its nonenablement argument on problems CFMT faced in setting up a commercial embodiment of the invention, the "beta tool Full Flow" machine. As noted before, CFMT had installed the Full Flow machine at a TI site. In its first runs, the machine did not meet TI's cleanliness standards. After months of experiments, the inventors identified the problem in a drying step and solved it. Concurrently, a patent application that led to the *'532 patent* was pending before the PTO. While prosecuting the application, CFMT submitted a list of advantages of the invention to the PTO, but did not tell the PTO of the problems at TI. The examiner allowed the case and the *'532 patent* issued. As also noted, the inventors eventually filed a patent application on the improvement [**7] that solved the problem. That application matured into *U.S. Patent No. 4,911,761* (the *'761 patent*).

On April 5, 2000, the district court granted YieldUp's motion for summary judgment that the '532 and *'123 patents* were invalid for nonenablement. The district court construed the claims of the *'532 patent* as limited by the preamble terms "cleaning" and "treatment," which the district court construed to mean removing contaminants from the wafer surface. *CFMT, Inc., 92 F. Supp. 2d at 371-72*. Similarly, the district court construed the claims of the *'123 patent* as limited by the preamble term "wet processing," which the district court construed to mean the same as "cleaning." *Id. at 374*. The district court stated that the specification "must enable one skilled in the art to clean semiconductor wafers using the Full Flow system." *Id. at 377*. The district court found that "the Full Flow system that was based [*1337] on the '532 and *'123 patents* could not clean" wafers, that the "inventors experimented with the Full Flow system for more than six months," and "that the solution to the problem eventually resulted in the *'761 patent* demonstrates that [**8] the experimentation required . . . was not routine." *Id.*

The district court further conducted a bench trial to determine whether CFMT committed inequitable conduct in prosecuting the application that matured into the '523 patent. On June 6, 2001, the district court entered judgment that the '532 and *'123 patents* are unenforceable due to inequitable conduct before the PTO. The district court relied on two events during prosecution of the application leading to the parent *'532 patent*. First, CMFT did not report to the PTO the initial TI test results (the TI data). The court concluded that the data was material because "a reasonable examiner would have considered data rebutting [the invention's] advantages in deciding whether to allow" the patents. *CFMT, Inc., 144 F. Supp. 2d at 317*. Second, during prosecution, the applicants traversed an obviousness rejection and stated eleven advantages of the invention. The district court found that the undisclosed TI data contradicted these laudatory statements. Because it considered the TI data highly material, the district court inferred that CMFT intended to deceive the PTO.

The district court then entered final judgment [**9] that the claims of the '532 and *'123 patents* were invalid and unenforceable. CFMT appealed to this court, which has jurisdiction under *28 U.S.C. § 1295(a)(1) (2000)*.

II.

[HN1] This court reviews without deference a district court's grant of summary judgment. *Pitney Bowes, Inc. v. Hewlett-Packard Co., 182 F.3d 1298, 1309 (Fed. Cir. 1999)*. [HN2] A court considering summary judgment must "view the evidence presented through the prism of the substantive evidentiary burden." *Anderson v. Liberty Lobby, Inc., 477 U.S. 242, 245, 91 L. Ed. 2d 202, 106 S. Ct. 2505 (1986)*. The court must also draw all reasonable inferences in favor of the nonmovant. *Id. at 255*.

[HN3] This court also reviews claim construction without deference. *Cybor Corp. v. FAS Techs., Inc., 138 F.3d 1448, 1454 (Fed. Cir. 1998) (en banc)*. [HN4] Enablement is a question of law with factual underpinnings; this court reviews the ultimate legal conclusion without deference. *Plant Genetic Sys., N.V. v. DeKalb Genetics Corp., 315 F.3d 1335, 1339 (Fed. Cir. 2003)*. [HN5] This court reviews a determination of inequitable conduct for abuse of discretion and reviews the underlying factual [**10] issues of materiality and intent for clear error. *Bristol-Myers Squibb Co. v. Rhone-Poulenc Rorer, Inc., 326 F.3d 1226, 1234 (Fed. Cir. 2003)*.

A. Nonenablement

The district court based its nonenablement judgment on two grounds: (1) lack of utility or inoperability and (2) undue experimentation needed to carry out the invention. The district court first construed each of the preamble terms "cleaning," "treatment," and "wet processing" as requiring "removal of contaminants." Based on that construction, the district court concluded that "the claims of the '532 and *'123 patents* must enable one skilled in the art to clean semiconductor wafers using the Full Flow system." The district court considered that "the first wafers processed with the Full Flow system appeared clean to the naked eye" but looked "filthy" viewed using laser scanning. The district court concluded that the TI data showed that the claimed system did not remove particles until the inventors developed the [*1338] improvements leading to the '716 patent. The district court found that "the Full Flow system that was based on the '532 and *'123 patents* could not clean semiconductor wafers." The district court considered [**11] that the inventors experimented "for more than six months" making "hundreds of modifications." The district court concluded that the "fact that the solution to the problem eventually resulted in the *'761 patent* demonstrates that the experimentation required to enable the '532 and *'123 patents* was not routine."

The parties do not challenge the district court's construction of the preamble terms "cleaning," "treatment," and "wet processing" as a limitation requiring "removal of contaminants." The parties also do not dispute that the record shows CFMT's initial efforts to build the claimed apparatus and to carry out the individual steps of the claimed method required undue experimentation. Instead, this case asks this court to examine whether these claims required a specific level of contaminant removal that the disclosure did not enable. Further, this court must consider whether the improvements in the '716 patent show that the '532 and *'123 patents* did not enable the scope of those claimed inventions.

At the outset, the district court erred in requiring that the patent disclosures enable a single embodiment, the Full Flow system, to meet TI's commercial standards. In essence, the [**12] district court set the enablement bar too high. [HN6] Enablement does not require an inventor to meet lofty standards for success in the commercial marketplace. Title 35 does not require that a patent disclosure enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment absent a claim limitation to that effect.

Title 35 requires only that the inventor enable one of skill in the art to make and use the full scope of the claimed invention. Thus, when an invention claims a general system to improve the cleaning process for semiconductor wafers, the disclosure enables that invention by showing improvements in the overall system. *See, e.g., Engel Indus., Inc. v. Lockformer Co., 946 F.2d 1528, 1533 (Fed. Cir. 1991)* ("The enablement requirement is met if the description enables any mode of making and using the claimed invention."). Of course, if a patent claimed a system that achieved cleanliness up to a specified numerical particle-free range, then enablement would require disclosure of a method that enables one of ordinary skill to achieve that range without undue experimentation. Thus, the level of disclosure necessary to satisfy [**13] *section 112 of title 35* varies according to the scope of the claimed invention. *Durel Corp. v. Osram Sylvania Inc., 256 F.3d 1298, 1306-07 (Fed. Cir. 2001)*; *In re Wright, 999 F.2d 1557, 1561 (Fed. Cir. 1993)*; *In re Wands, 858 F.2d 731, 737 (Fed. Cir. 1988).*

The claims of the '532 and *'123 patents* state no standard of cleaning. As the district court correctly found, "cleaning" in the context of this invention means generally removing contaminants from the wafer surface. Absent some standard for cleanliness in the claims, this court proceeds to examine the record for a showing that the disclosures of the CFMT patents would enable a person of skill in the art to make and use a system or apparatus to achieve any level of contaminant removal without undue experimentation. *See Engel Indus., 946 F.2d at 1533.*

The record contains evidence that the inventors' prototype removed grease stains. The inventors testified that before setting up the TI apparatus, they verified by naked eye that a prototype of the invention removed penciled grease marks. This record evidence is probative of whether [*1339] the "removal of contaminants" [**14] limitation is enabled. This court also notes that the record contains no evidence that a person of ordinary skill would have to undertake undue experimentation to build a similar prototype and carry out the claimed method to remove the contaminants -- in this instance, grease marks.

The lengthy experiments at TI do not show nonenablement because the inventors undertook that work to satisfy TI's particular commercial requirements, not to show enablement of the scope of the claimed inventions. [HN7] "Patents are not production documents, and nothing in the patent law requires that a patentee must disclose data on how to mass-produce the invented product. . . . The law requires that patents disclose inventions, not mass-production data, and that patents enable the practice of inventions, not the organization and operation of factories." *Christianson v. Colt Indus. Operating Corp., 822 F.2d 1544, 1562 (Fed. Cir. 1987).* Reliance on the TI data alone also betrays another error, namely that this [HN8] court gauges enablement at the date of the filing,

not in light of later developments. *In re Wright, 999 F.2d 1557, 1563 n.8 (Fed. Cir. 1993).*

The district court essentially [**15] concluded that the invention claimed in the patents at issue simply did not work, that is, could not clean wafers, and therefore it would require undue experimentation to carry out the invention. *See 35 U.S.C. § 101 (2000).* [HN9] This court has recognized the relationship between the enablement requirement of *§ 112* and the utility requirement of *§ 101. See, e.g., In re Swartz, 232 F.3d 862, 863 (Fed. Cir. 2000)* ("If the claims in an application fail to meet the utility requirement because the invention is inoperative, they also fail to meet the enablement requirement because a person skilled in the art cannot practice the invention"); *EMI Group N. Am., Inc. v. Cypress Semiconductor Corp., 268 F.3d 1342, 1348 (Fed. Cir. 2001).* In this case, however, the district court similarly set the standard for utility too high for this invention. While the district court's major premise is correct that [HN10] an inoperable invention is not enabled, the district court erred in its minor premise that the claimed invention is inoperable and lacks utility.

[HN11] The inoperability standard for utility applies primarily to claims with impossible limitations. [**16] *See, e.g., Process Control Corp. v. HydReclaim Corp., 190 F.3d 1350, 1359 (Fed. Cir. 1999)* (claims found inoperable because they require violating the principle of conservation of mass); *Newman v. Quigg, 877 F.2d 1575 (Fed. Cir. 1989)* (claims to a perpetual motion machine ruled inoperable). Moreover, where a patent discloses several alternative combinations of methods (as most systems claims will), the party asserting inoperability must show that all disclosed alternatives are inoperative or not enabled. *EMI Group, 268 F.3d at 1349.* The '532 and *'123 patents* do not claim an impossible result or an inoperative invention.

Because the preamble term "cleaning" means only "removal of contaminants," not removal of all contaminants or removal of contaminants according to the TI commercial standard, the inventor shows utility and enables the invention by disclosing "removal of contaminants." Even if the single Full Flow embodiment does not achieve complete cleaning, that alone would not render the invention inoperative. *See Moleculon Research Corp. v. CBS, Inc., 793 F.2d 1261, 1269* (claims had utility despite only a partial description of [**17] how to reach the claimed goal of "restoring a preselected pattern" in a puzzle; it sufficed to describe a general approach to solving the puzzle); *EMI Group, 268 F.3d at 1349.* Nor would it render the claims invalid as nonenabled. [*1340] *See Engel Indus., 946 F.2d at 1533.* In this case, with its specific claims and invention, the specification needed to teach one of ordinary skill to make and use a system or apparatus that removes any contaminants. In sum, any meaningful "cleaning" would satisfy the claimed goal of "cleaning of semiconductor wafers."

The district court's second ground for nonenablement invoked the '761 improvement patent as evidence that the inventors engaged in undue experimentation to "clean" semiconductor wafers. The district court reasoned that the inventor had not enabled the '532 and *'123 patents* because only the further invention of the '761 improvement patent sufficed to meet TI's commercial standard.

[HN12] Improvement and selection inventions are ubiquitous in patent law; such developments do not alone cast doubt on enablement of the original invention. *See Hormone Research Found., Inc. v. Genentech, Inc., 904 F.2d 1558, 1568 (Fed. Cir. 1990)* [**18] (citing *In re Hogan, 559 F.2d 595 (CCPA 1977)).* In general, few patented inventions are an immediate commercial success. Rather, most inventions require further development to achieve commercial success. Thus, additional inventive work does not alone show nonenablement.

Moreover, the district court's reasoning presumes incorrectly that development of an improvement patent, the '761 in this case, implies extensive experimentation. To the contrary, [HN13] patent acquisition does not require any threshold level of effort or ingenuity. *See 35 U.S.C. § 103(a) (2000)* ("Patentability shall not be negatived by the manner in which the invention was made."); *35 U.S.C. § 103* Revision Notes and Legislative Reports, 1952 Notes ("It is immaterial whether [the invention] resulted from long toil and experimentation or from a flash of genius."); *Life Techs., Inc. v. Clontech Labs., Inc., 224 F.3d 1320, 1325 (Fed. Cir. 2000)* (stating that "the path that leads an inventor to the invention is expressly made irrelevant to patentability by statute"). Thus, the '761 improvement patent alone is not conclusive evidence of undue [**19] experimentation.

Because the district court misapplied the law of enablement in concluding that the claims of the '532 and *'123 patents* are invalid, this court vacates that part of the decision. While the record at this stage does not appear to present a genuine issue of material fact about whether a person of ordinary skill in the art could achieve any level of cleaning with the claimed invention without undue experimentation, this court remands for the district court to reconsider that question. The district court may decide, under the correct legal standard, whether to grant CFMT's cross-motion for summary judgment of enablement or whether to proceed to trial on that issue.

B. Inequitable Conduct

[HN14] Inequitable conduct requires proof that a patent applicant did not disclose material information to

the PTO with intent to deceive. *Kingsdown Med. Consultants, Ltd. v. Hollister Inc., 863 F.2d 867, 872 (Fed. Cir. 1988)*. More specifically, "inequitable conduct includes affirmative misrepresentation of a material fact, failure to disclose material information, or submission of false material information, coupled with an intent to deceive." *Molins PLC v. Textron, Inc., 48 F.3d 1172, 1178 (Fed. Cir. 1995)*. [**20] These elements must be shown with clear and convincing evidence. *Id.* The district court applied the pre-1992 standard for materiality, because the relevant acts took place before 1992. Under that standard, information is material if "there is a substantial likelihood that a reasonable examiner would consider it important in deciding whether to allow the application to issue as a patent." See *Molins, 48 F.3d at 1179 n.8.*

[*1341] The district court concluded that CFMT committed inequitable conduct in its comments about the advantages of the invention during prosecution of the *'532 patent* to overcome a rejection for obviousness and in its failure to disclose the TI data. The district court inferred intent based on the inventors' knowledge of the materiality of the comments and omissions.

1. Misrepresentations to the PTO

The district court found that the applicants, in traversing an obviousness rejection during prosecution before the PTO, misrepresented the invention by stating its advantages without disclosing the TI data. The statements at issue appear in an amendment filed in December 1988 (emphases added):

> The *new and/or unexpected results and advantages* [**21] achieved by the presently claimed invention include:
>
> 1. reduction of *contamination by airborne particles*;
>
> 2. reduction of *contamination from human or robotic operators*;
>
> 3. good heat transfer between process chemicals and wafers;
>
> 4. uniform exposure of the wafers to reagent chemicals at uniform concentrations for precisely limited periods of time;
>
> 5. reduction of hazards to personnel by minimizing exposure to chemicals;
>
> 6. minimizing stagnant conditions and avoiding filming effects; and
>
> 7. providing a mechanically simple process and apparatus which allow for easy operation and cleaning while minimizing the possibility [sic] contaminant build-ups in the apparatus.
>
> Still further advantages are provided by preferred embodiments of the present invention, including:
>
> 1. the reduction of quantities of hazardous process fluids used due to recirculation of the process fluids;
>
> 2. the ability to provide quality drying fluids to displace the residual rinsing fluid;
>
> 3. the ability to provide a high-quality rinsing fluid having both low suspended solids and low dissolved impurities; and
>
> 4. the ability to provide high flow rates of rinsing fluid to rinse the wafers and precisely [**22] dilute concentrated chemical reagents.
>
> *The net effect of all the above advantages is to reduce the risk of introducing contaminants* while simultaneously improving the yield of non-defective semiconductor devices.

The district court found that an examiner would have considered the TI data important because it rebuts those stated advantages. The district court focused on the final sentence quoted above, treating it as a "summary of the advantages distinguishing the Full Flow system from the Aigo tool" and interpreting "contaminants" to include all undesirable materials mentioned in the enumerated advantages. The district court concluded that "the inventors' statements in response to the obviousness rejection were inaccurate and constituted a misrepresentation."

The district court clearly erred in finding that the applicants' statements were material misrepresentations. In the first place, the statements were not inaccurate. As recognized by the examiner in the Notice of Allowance, the invention advances the art by closing the system for cleaning semiconductor wafers. A closed system provides the inherent advantage of less contamination by airborne particles.

The final [**23] sentence of the applicants' advantages advocacy refers to reducing contaminants as described in the enumerated examples. Moreover, the only specific contaminants in those examples are airborne particles (the only mention of "particles") [*1342] and contaminants from human or robotic operators. Thus, the advantages advocacy recited only the natural, expected

results of a closed system. The final quoted sentence at most overemphasizes the benefits of the invention. This advocacy does not rise to the level of misrepresentation.

The district court also clearly erred in finding that the examiner relied on the applicants' advantages advocacy. To the contrary, the examiner, in supplying reasons for allowance, stated only that the art of record does not teach a closed, hydraulic system as claimed. Thus, the examiner concluded that no combination of the prior art, even if supported by a motivation to combine, would disclose all the limitations of the claims. In other words, the examiner detected, in light of all limitations of the claims, no obviousness. *See In re Gulack, 703 F.2d 1381, 1385 n.9 (Fed. Cir. 1983)*; *In re Royka, 490 F.2d 981, 985 (CCPA 1974)* [HN15] (obviousness [**24] requires a suggestion of all limitations in a claim). Therefore the examiner did not appear to resort to consideration of secondary considerations, such as the unexpected results and advantages in the quoted statements, to surmount the obviousness objection. In sum, the advantages advocacy was not as highly material as the district court seemed to think.

[HN16] An applicant cannot prove unexpected results with attorney argument and bare statements without objective evidentiary support. *See In re Lindner, 457 F.2d 506, 508, 59 C.C.P.A. 920 (CCPA 1972)*; *In re Geisler, 116 F.3d 1465 (Fed. Cir. 1997)* ("attorney argument [is] not the kind of factual evidence that is required to rebut a prima facie case of obviousness"); *In re Soni, 54 F.3d 746, 750 (Fed. Cir. 1995)* ("It is well settled that unexpected results must be established by factual evidence. Mere argument or conclusory statements . . . [do] not suffice." (quoting *In re De Blauwe, 736 F.2d 699, 705 (Fed. Cir. 1984)*). During prosecution, an applicant may submit objective factual evidence to the PTO in the form of patents, technical literature, and declarations under *37 C.F.R. § 1.132* [**25] *(2003)* submitting expert testimony and, at times, test data. The advantages advocacy in this case does not fit any of these categories and was unaccompanied by and not asserted to be supported by any factual evidence. Therefore, a reasonable examiner would not have found it important in deciding whether to allow the application. Instead, the examiner expressly stated the grounds for allowance, namely that the art of record does not teach a closed, hydraulic system as claimed.

In sum, the district court clearly erred in finding that the applicants' statements to the PTO were misrepresentations and in finding that those statements were highly material to the examiner's actions.

2. Failure to disclose the TI data

The district court also concluded that CFMT breached the duty of candor because it did not disclose the TI data to the PTO. The district court considered the TI data material to enablement.

As already noted, the TI data was temporally and substantively of very marginal relevance to enablement of the claims as filed. As noted, the TI data reflects a commercial, not a statutory, standard for enablement. The district court therefore clearly erred in concluding that the TI data [**26] was highly material.

3. Intent

This court recognizes that [HN17] a district court may infer intent to deceive the PTO. However, even gross negligence does not alone suffice to establish intent. *Kingsdown Med. Consultants, Ltd. v. Hollister Inc., 863 F.2d 867, 876 (Fed. Cir. 1988)*. Instead, "the involved conduct, viewed in light of all the evidence, including [*1343] evidence indicative of good faith, must indicate sufficient culpability to require a finding of intent to deceive." *Id.*

This court discerns no evidence that CFMT intended to deceive the PTO. As explained above, the materiality of the undisclosed subject matter is low. Therefore, the trial court had little basis for inferring intent. The district court clearly erred in finding that the applicants intentionally withheld material information and therefore abused its discretion in concluding that the applicants engaged in inequitable conduct in prosecuting the patents at issue.

Accordingly, this court reverses the district court's decision on inequitable conduct.

CONCLUSION

The district court erred in granting summary judgment that the patents at issue do not meet the enablement requirement of *35 U.S.C. § 112* [**27] and in ruling after trial that the patents are unenforceable due to inequitable conduct before the PTO. This court therefore reverses-in-part, vacates-in-part, and remands.

COSTS

Each party shall bear its own costs.

REVERSED-IN-PART, VACATED-IN-PART, and REMANDED

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LEXSEE 490 F.2D 981

IN THE MATTER OF THE APPLICATION OF STEPHEN F. ROYKA AND ROBERT G. MARTIN

Patent Appeal No. 9092

UNITED STATES COURT OF CUSTOMS AND PATENT APPEALS

490 F.2d 981; 1974 CCPA LEXIS 200; 180 U.S.P.Q. (BNA) 580

February 7, 1974, Decided.

PRIOR HISTORY: [**1] Serial No. 648,701.

CASE SUMMARY:

PROCEDURAL POSTURE: Appellants sought review of a decision of the U.S. Patent Office Board of Appeals that affirmed the rejection of appellants' patent claims for a printed matter self-testing system as obvious under *35 U.S.C.S. § 103* and anticipated under *35 U.S.C.S. § 102.*

OVERVIEW: Appellants designed an answer sheet for use in self-testing that featured a response area having legible, confusing information in erasable printing imposed over answers in permanent printing. The claims were rejected as anticipated by prior art under *35 U.S.C.S. § 102* and as obvious under *35 U.S.C.S. § 103.* The court reversed, finding that the superimposed printing in appellant's response was legible and imparted information, which was not the case in the prior references. Therefore, the court held that a finding of anticipation was not warranted, as the claimed invention was not disclosed in the prior art. The court also noted that printed matter could constitute structural limitations upon which patentability could be predicated. The court found that the claims were not obvious for the same reasons they were not anticipated by prior art.

OUTCOME: The court reversed the Board's decision that rejected appellants' claims as obvious and anticipated. The court held that appellants' invention was sufficiently distinguished from prior art that it was neither anticipated by it nor obvious in light of it.

LexisNexis(R) Headnotes

Patent Law > Anticipation & Novelty > General Overview
Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals
[HN1] To support an anticipation rejection, all elements of the claim must be found in the reference.

Patent Law > Claims & Specifications > General Overview
Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals
Patent Law > U.S. Patent & Trademark Office Proceedings > General Overview
[HN2] Claims are not to be read in a vacuum and while it is true they are to be given the broadest reasonable interpretation during prosecution, their terms still have to be given the meaning called for by the specification of which they form a part.

Patent Law > Anticipation & Novelty > Elements
Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals
[HN3] Anticipation requires a finding that the claimed invention be disclosed by prior art.

Patent Law > Jurisdiction & Review > Subject Matter Jurisdiction > Appeals
Patent Law > Subject Matter > Products > General Overview
Patent Law > U.S. Patent & Trademark Office Proceedings > General Overview
[HN4] Printed matter may very well constitute structural limitations upon which patentability can be predicated.

OPINION BY: RICH

OPINION

[*981] RICH, Judge.

This appeal is from the decision of the Patent Office Board of Appeals affirming the examiner's rejection of claims 28 and 30-36 of application serial No. 648,701, filed June 26, 1967, entitled "Responsive Answer System." We reverse.

The Invention

The appealed claims are directed to a device in the nature of an answer sheet for use in self-instruction and testing. The answer sheet may be associated with questions or separate therefrom. the essential features of the invention are that there are printed on the answer sheet in "response areas" meaningful information in. permanent printing and confusing information in printing which can be removed, as by an eraser, both being legible so that a student, seeing a choice of answers to a question, must make a selection. Having made a selection, he then applies as eraser to the selected response area and some of the information will be readily removed. What remains advises him of the correctness or otherwise of his answer. The following figures from the drawings are illustrative:

[Graphic omitted. See illustration in original.]

Fig. 1A shows two response areas [**2] to a given question before any removing action [*982] by the student has taken place and Fig. 1B shows the permanent information remaining in each after erasure of the removable information. Of course, if the student makes an initial choice of area A, showing up "YES" or some other indication of a correct answer, he will not need to proceed further and erase the B area. In a modified form of the invention, a wrong selection, plus erasure, may expose, instead of or in addition to a statement that the answer is wrong, a number or other reference to further material which is to be studied.

A preferred method of printing the permanent meaningful information and the removable confusing information is by that type of xerography in which a fusible toner is used, the permanence of the printing depending on the extent to which the toner image is "fixed" or fused by heat. By successive printings of the two kinds of information with fixing to different degrees, one image can be made permanent and the other made subject to easy removal, both images retaining such similarity of appearance that the user of the answer sheet cannot tell them apart.

Claim 28 is the principal claim, all [**3] others being dependent thereon, and reads as follows:

28. A device for selectively indicating information comprising

a support having response areas for presenting information for selection,

permanent printing indicative of meaningful information permanently fixed to said support within a response area, and

removable printing indicative of confusing information removably fixed to said support within a response area,

said meaningful and confusing information being substantially legible even when said permanent and removable printing are fixed over one another on said support,

said permanent and removable printing being substantially similar such that an observer cannot determine which information is permanent and which is removable

whereby the information within a response area is selected by attempting to remove the printing therein with the failure to remove printing identifying meaningful information.

Claims 30-36 add limitations which need not be considered except for noting that claims 33 and 34 alone specify the use of a xerographic toner, for which reason they were rejected on a different ground from the other claims.

The Rejection

The following references [**4] were relied on:

[SEE TABLE IN ORIGINAL]

Claims 28, 30, 31, and 32 were rejected as anticipated under *35 USC 102* by Bernstein; claims 28, 31, 32, 35, and 36 were rejected as anticipated under *§ 102* by Reid; and claims 33 and 34 were rejected under *35 USC 103* for obviousness, on either Bernstein or Reid in view of Lein. These were the examiner's rejections and the board affirmed them, adhering to its decision on reconsideration.

Bernstein discloses an answer sheet in which printed information representing a response is "temporarily concealed from the observer" and he discloses a number of different ways of effectively concealing the response. His specification states:

The objects of the invention are accomplished by utilizing the hiding media to confuse the participant and to render the response and the hiding media indistinguishable and thus conceal the presence, absence, nature or position of the response from the participant. This may be effectuated by careful attention being paid to a

number of factors including the design, [*983] color and position of the hiding or confusing media.

Fig. 1 of Bernstein's drawings illustrates some of his concealing means:

[**5] [Graphic omitted. See illustration in original.]

The following is the written description:

Referring now to the drawing, FIG. 1 illustrates some of the many optically confusing patterns which may be positioned between the printed structure to be concealed and the point of observation. Column 11 shows the information which is to be concealed. This information is repeated in columns 12 through 16 but in each case is concealed by a pattern in accordance with the present invention. Column 12 utilizes a pattern comprising an alphabetical maze in both line and half tone screen. Column 13 utilizes a pattern comprising an absorbing field having a plurality of irregular dot-like interstices. Column 14 utilizes a pattern comprising a maze of plus signs combined with dots. Columns 15 and 15 illustrate irregular and non-repetitious patterns. Bernstein says that if at least 50% of the response is actually covered by the opaque portions of the confusion pattern, complete concealment is obtained. He also says that added means of concealment may be used, such as scoring and embossing and perforating the paper in order to scatter the light or let it shine through.

Reid is entitled [**6] "Transformation Picture and Print." The invention is said to be useful for advertisements, Christmas cards, birthday cards, valentines, and the like and as a source of amusement and instruction for children. It consists of a picture or print, part of which is permanently printed and part of which is removable from the paper on which it is printed. For the latter various soluble undercoatings or inks are described. If the picture is washed with a solvent, which may be water, the removable part disappears and the pictorial and/or typographic matter changes. The invention is illustrated by a typical nineteenth century temperance propaganda piece depicting the evils of drink. In the finished picture there are three scenes from left to right: Scene 1, the innocent child leads her father home from the pub; Scene 2, Father sits slumped in the kitchen chain with his bottle beside him, the family wash hanging above his head, this picture being entitled "The Effects of Drink"; Scene 3, Mother stands in front of a sign reading "Pawn Shop." Across the bottom of the picture is a legend which says "Wash the above and see what water will do." Fig. II shows the result of washing with water: Scene [**7] 1, a handsome young man and his happy daughter stroll on the street; Scene 2, Father sits erect in a well-appointed room at a cloth-covered table, apparently having a cup of tea, obviously a gentleman; Scene 3, Mother beams from the sideline and the Pawn Shop sign has vanished. Two new subscriptions appear and the words "The" and "Drink" have disappeared, the resultant being a new picture title reading "The Beneficial Effects of Temperance." "The Beneficial" and "Temperance" were covered by some soluble opaque in the original picture. No doubt the overall effect is instruction. Perhaps there was amusement in bringing about the transformation.

Lein relates to xerography and is relied on only for its disclosure of the removability of partially fused toner and the permanence of fully fused toner.

OPINION

As to the *§ 102* anticipation rejections, it will suffice to consider independent claim 28. If it is not fully met by Reid [*984] or Bernstein, neither are the more limited dependent claims. It is elementary that [HN1] to support an anticipation rejection, all elements of the claim must be found in the reference. We do not find claim 28 anticipated by Bernstein because, as [**8] we read the claim, it requires the display of legible meaningful and legible confusing information simultaneously, between which the user of the device may make a selection before he undertakes to remove any of the information from the response area selected by him. The element we find most clearly missing, contrary to the reasoning of the examiner and the board, is the legible confusing information. The Patent Office proposes to read this limitation on Bernstein's confusion patterns which are nothing but meaningless obscuring screens, conveying no information and providing the user with no basis for making a selection, as called for by claim 28. In appellants' device the legible confusing information - i.e., the wrong answers - are legible in the sense that they can be read as intelligible words, not merely a jumble of type serving to obscure the words of the wrong answers.

Appellants were fully aware of Bernstein and discussed its disclosures in their specification, distinguishing from this and other prior art, saying, in part:

The inventive concept hereof confuses not by physical blocking as taught by the prior art, but by compounding, associating (including disarranging) [**9] permanent information with confusing information, usually at least some of which is similar in character to the permanent information as to render it impossible to tell which is permanent and which is removable confusing information. In the invention, generally no attempt is made to designedly physically cover the permanent information, but to confuse it beyond interpretation by the presentation of extraneous removable, confusing information.[HN2]

Claims are not to be read in a vacuum and while it is true they are to be given the broadest reasonable interpre-

tation during prosecution, their terms still have to be given the meaning called for by the specification of which they form a part. We cannot read the terms "legible" and "information" on Bernstein's confusion patterns, as did the examiner and the board. They are not "legible," as appellants use the term, and they convey no information.

As to anticipation by Reid, we find neither appellants' basic concept nor the substance of claim 28 to be disclosed.Apparently the solicitor could find little to support the rejection in Reid for all he says in his brief - so far as claim 28 is concerned - is:

Reid discloses a sheet which may [**10] be used for instruction and which may have a removable design partly covering a fixed design * * *. Therefore, the disclosure of the reference encompasses the arrangement wherein a removable design covers a fixed design with both designs being substantially legible.

But claim 28 does not call for an arrangement wherein a removable design covers a fixed design. It calls for response areas, which Reid does not have, containing meaningful information in permanent printing together with removable printing conveying confusing information, both legible at the same time, between which a "selection" can be made. The only choice offered to the user by Reid is to follow the instruction to wash the whole visible picture with water or other solvent, thus removing the over-printing, to discover what the permanent picture is. The Patent Office attempt to read claim 28 on this reference is a tour de force. We hold that Reid does not anticipate for failure to meet the limitations of claim 28 to "response areas," to the presentation of two categories of information (meaningful-permanent and removable-confusing) within such areas, and the possibility of selection. [HN3] Anticipation requires a finding [**11] that the claimed invention be disclosed. It is not enough to say that appellants' invention and the reference are [*985] both usable for instruction and both consist of permanent and removable printings on paper, as did the solicitor.

The dependent claims rejected with claim 28, as anticipated under *§ 102*, are not anticipated since claim 28 is not anticipated. Some of them merely add features which are disclosed by the references and some do not.Insofar as they do not, they further negative anticipation. The examiner recognized this fact as to claims 33 and 34, which are limited to xerography, and therefore did not reject them under *§ 102*. Similarly, he did not reject claim 30 on Reid or claims 35 and 36 on Bernstein. We find that claims 35 and 36 contain limitations which additionally distinguish from Reid. We have already noted that Reid has no "response areas" as required by claim 28 and so Reid does not disclose the structure of claim 35 which additionally requires both the correct and incorrect answers to appear within the same response area.

As to claim 36, the examiner said it "is merely a printed matter variation of the design of the reference," Reid. This [**12] is not a valid reason for rejection. [HN4] Printed matter may very well constitute structural limitations upon which patentability can be predicated. We have commented on this matter in *In re Jones, 54 CCPA 1218, 373 F.2d 1007, 153 USPQ 77 (1967)*; and *In re Miller, 57 CCPA 809, 418 F.2d 1392, 164 USPQ 46 (1969)*, and will not repeat ourselves. The limitations of claim 36 are not remotely suggested by Reid.

There remains the *§ 103* rejection of claims 33 and 34. Do they, taken together with all of the limitations of claim 28 from which they depend, define obvious subject matter? The difference between claim 28 and these two dependent claims is that they add the limitations to xerography. If Bernstein and Reid showed the claimed invention except for xerography, the addition of the Lein reference would make the subject matter of the claims obvious. But that is not the situation here. Adding the knowledge of xerographic technology to Bernstein or Reid still does not make the invention of claims 33 and 34 obvious for the same reasons we have given above in discussing anticipation. The essence of appellants' invention, as set forth in claim 28, is still missing notwithstanding the addition [**13] of the Lein reference and we see nothing in the combinations of references which would have made the invention obvious to one of ordinary skill in the art at the time it was made. We will, therefore, reverse this rejection.

The decision of the board is reversed.

REVERSED

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LEXSEE 51 F.3D 1552

IN RE THOMAS F. DEUEL, YUE-SHENG LI, NED R. SIEGEL and PETER G. MILNER

94-1202

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

51 F.3d 1552; *1995 U.S. App. LEXIS 6200*; *34 U.S.P.Q.2D (BNA) 1210*

March 28, 1995, Decided

PRIOR HISTORY: [**1] Appealed from: U.S. Patent and Trademark Office Board of Patent Appeals and Interferences. (Serial No. 07/542,232).

DISPOSITION: REVERSED

CASE SUMMARY:

PROCEDURAL POSTURE: Applicants appealed from a decision of the United States Patent and Trademark Office Board of Patent Appeals and Interferences that affirmed a rejection of applicants' patent application for a process of isolating and making DNA molecules.

OVERVIEW: Applicants sought a patent for their process of isolating and manufacturing specific DNA molecules. The examiner rejected their application, and the Board of Patent Appeals (Board) affirmed. Applicants appealed, and the court reversed. The court held the Board erred by rejecting the application under the obviousness standard at *35 U.S.C.S. § 103*. The court agreed with applicants that the Board mistakenly found that the prior art suggested isolating and making the molecules specified in the application and thus were obvious. Rather, the prior art did not disclose the specified molecules; it would have been highly unlikely for one of ordinary skill and art in the field to achieve, based on common teaching, what was achieved by applicants. The fact that there was some similarity in the structure of applicant's molecules and those disclosed by prior art did not render applicants' molecules obvious.

OUTCOME: The decision was reversed on the grounds that the Board of Patent Appeals erred by finding applicants' process obvious. Prior art did not disclose the molecules described in applicants' patent application.

LexisNexis(R) Headnotes

Civil Procedure > Appeals > Standards of Review > De Novo Review
Patent Law > Nonobviousness > Evidence & Procedure > Fact & Law Issues
Patent Law > U.S. Patent & Trademark Office Proceedings > General Overview

[HN1] Obviousness is a question of law, which the appeals court reviews de novo, though factual findings underlying the Board of Patent Appeals' obviousness determination are reviewed for clear error.

Patent Law > Nonobviousness > Elements & Tests > General Overview

[HN2] The examiner bears the burden of establishing a prima facie case of obviousness.

Patent Law > Nonobviousness > Elements & Tests > General Overview

[HN3] Only if the examiner meets his burden of establishing a prima facie case of obviousness does the burden of coming forward with rebuttal argument or evidence shift to the applicant.

Patent Law > Nonobviousness > Elements & Tests > General Overview
Patent Law > U.S. Patent & Trademark Office Proceedings > Appeals

[HN4] When the references cited by the examiner fail to establish a prima facie case of obviousness, the rejection is improper and will be overturned.

Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
Patent Law > Nonobviousness > Elements & Tests > Prior Art
Patent Law > Nonobviousness > Evidence & Procedure > Prima Facie Obviousness
[HN5] Normally a prima facie case of obviousness is based upon structural similarity, i.e., an established structural relationship between a prior art compound and the claimed compound.

Patent Law > Anticipation & Novelty > General Overview
Patent Law > Nonobviousness > Elements & Tests > General Overview
Patent Law > Subject Matter > Processes > General Overview
[HN6] Structural similarity between claimed and prior art subject matter, where the prior art gives reason or motivation to make the claimed compositions, creates a prima facie case of obviousness.

Patent Law > Nonobviousness > Elements & Tests > General Overview
Patent Law > Subject Matter > Processes > General Overview
[HN7] A broad genus does not necessarily render obvious each compound within its scope.

Patent Law > Anticipation & Novelty > General Overview
Patent Law > Nonobviousness > Elements & Tests > General Overview
Patent Law > Subject Matter > Processes > General Overview
[HN8] The existence of a general method of isolating cDNA or DNA molecules is essentially irrelevant to the question whether the specific molecules themselves would have been obvious, in the absence of other prior art that suggests the claimed DNAs.

Patent Law > Nonobviousness > Elements & Tests > General Overview
Patent Law > Subject Matter > Processes > General Overview
[HN9] The fact that one can conceive a general process in advance for preparing an undefined compound does not mean that a claimed specific compound was precisely envisioned and therefore obvious.

COUNSEL: G. Harley Blosser, Senniger, Powers, Leavitt & Roedel, of St. Louis, Missouri, argued for appellants. With him on the brief was Donald G. Leavitt.

Donald S. Chisum, Morrison & Foerster, of Seattle, Washington, argued for Amicus Curiae, The Biotechnology Industry Association and The Bay Area Bioscience Center. With him on the brief were Debra A. Shetka, Morrison & Forester, of Palo Alto, California and Robert P. Blackburn, of Emeryville, California.

Teddy S. Gron, Acting Associate Solicitor, of Arlington, Virginia, argued for appellee. With him on the brief was Albin F. Drost, Acting Solicitor. Nancy J. Linck, Office of the Solicitor, of Arlington, Virginia, represented appellee.

JUDGES: Before ARCHER, Chief Judge, NIES and LOURIE, Circuit Judges.

OPINION BY: LOURIE

OPINION

[*1553] LOURIE, *Circuit Judge.*

Thomas F. Deuel, Yue-Sheng Li, Ned R. Siegel, and Peter G. Milner (collectively "Deuel") appeal from the November 30, 1993 decision of the U.S. Patent and Trademark Office Board of Patent Appeals and Interferences affirming the examiner's final rejection of [**2] claims 4-7 of application Serial No. 07/542,232, entitled "Heparin-Binding [*1554] Growth Factor," as unpatentable on the ground of obviousness under *35 U.S.C. § 103 (1988). Ex parte Deuel, 33 USPQ2d 1445 (Bd. Pat. App. Int. 1993).* Because the Board erred in concluding that Deuel's claims 5 and 7 directed to specific cDNA molecules would have been obvious in light of the applied references, and no other basis exists in the record to support the rejection with respect to claims 4 and 6 generically covering all possible DNA molecules coding for the disclosed proteins, we reverse.

BACKGROUND

The claimed invention relates to isolated and purified DNA and cDNA molecules encoding heparin-binding growth factors ("HBGFs"). [1] HBGFs are proteins that stimulate mitogenic activity (cell division) and thus facilitate the repair or replacement of damaged or diseased tissue. DNA (deoxyribonucleic acid) is a generic term which encompasses an enormous number of complex macromolecules made up of nucleotide units. DNAs consist of four different nucleotides containing the nitrogenous bases adenine, guanine, cytosine, and thymine. A sequential grouping of three such nucleotides (a "codon") codes for [**3] one amino acid. A DNA's se-

quence of codons thus determines the sequence of amino acids assembled during protein synthesis. Since there are 64 possible codons, but only 20 natural amino acids, most amino acids are coded for by more than one codon. This is referred to as the "redundancy" or "degeneracy" of the genetic code.

1 For a more extensive discussion of recombinant DNA technology, *see In re O'Farrell, 853 F.2d 894, 895-99, 7 USPQ2d 1673, 1674-77 (Fed. Cir. 1988)*; *Amgen Inc. v. Chugai Pharmaceutical Co., 927 F.2d 1200, 18 USPQ2d 1016* (Fed. Cir.), *cert. denied, 502 U.S. 856 (1991).*

DNA functions as a blueprint of an organism's genetic information. It is the major component of genes, which are located on chromosomes in the cell nucleus. Only a small part of chromosomal DNA encodes functional proteins.

Messenger ribonucleic acid ("mRNA") is a similar molecule that is made or transcribed from DNA as part of the process of protein synthesis. Complementary DNA ("cDNA") is a complementary copy ("clone") [**4] of mRNA, made in the laboratory by reverse transcription of mRNA. Like mRNA, cDNA contains only the protein-encoding regions of DNA. Thus, once a cDNA's nucleotide sequence is known, the amino acid sequence of the protein for which it codes may be predicted using the genetic code relationship between codons and amino acids. The reverse is not true, however, due to the degeneracy of the code. Many other DNAs may code for a particular protein. The functional relationships between DNA, mRNA, cDNA, and a protein may conveniently be expressed as follows:

genomic		
DNA	mRNA	protein
	cDNA	other DNAs

Collections ("libraries") of DNA and cDNA molecules derived from various species may be constructed in the laboratory or obtained from commercial sources. Complementary DNA libraries contain a mixture of cDNA clones reverse-transcribed from the mRNAs found in a specific tissue source. Complementary DNA libraries are tissue-specific because proteins and their corresponding mRNAs are only made ("expressed") in specific tissues, depending upon the protein. Genomic DNA ("gDNA") libraries, by contrast, theoretically contain all of a species' chromosomal DNA. The molecules present in cDNA and DNA libraries may be [**5] of unknown function and chemical structure, and [*1555] the proteins which they encode may be unknown. However, one may attempt to retrieve molecules of interest from cDNA or gDNA libraries by screening such libraries with a gene probe, which is a synthetic radiolabelled nucleic acid sequence designed to bond ("hybridize") with a target complementary base sequence. Such "gene cloning" techniques thus exploit the fact that the bases in DNA always hybridize in complementary pairs: adenine bonds with thymine and guanine bonds with cytosine. A gene probe for potentially isolating DNA or cDNA encoding a protein may be designed once the protein's amino acid sequence, or a portion thereof, is known.

As disclosed in Deuel's patent application, Deuel isolated and purified HBGF from bovine uterine tissue, found that it exhibited mitogenic activity, and determined the first 25 amino acids of the protein's N-terminal sequence. [2] Deuel then isolated a cDNA molecule encoding bovine uterine HBGF by screening a bovine uterine cDNA library with an oligonucleotide probe designed using the experimentally determined N-terminal sequence of the HBGF. Deuel purified and sequenced the cDNA molecule, which was [**6] found to consist of a sequence of 1196 nucleotide base pairs. From the cDNA's nucleotide sequence, Deuel then predicted the complete amino acid sequence of bovine uterine HBGF disclosed in Deuel's application.

2 Deuel determined that the N-terminal sequence of bovine uterus HBGF is Gly-Lys-Lys-Glu-Lys-Pro-Glu-Lys-Lys-Val-Lys-Lys-Ser-Asp-Cys-Gly-Glu-Trp-Gln-Trp-Ser-Val-Cys-Val-Pro.

Deuel also isolated a cDNA molecule encoding human placental HBGF by screening a human placental cDNA library using the isolated bovine uterine cDNA clone as a probe. Deuel purified and sequenced the human placental cDNA clone, which was found to consist of a sequence of 961 nucleotide base pairs. From the nucleotide sequence of the cDNA molecule encoding human placental HBGF, Deuel predicted the complete amino acid sequence of human placental HBGF disclosed in Deuel's application. The predicted human placental and bovine uterine HBGFs each have 168 amino acids and calculated molecular weights of 18.9 kD. Of the 168 amino acids present [**7] in the two HBGFs discovered by Deuel, 163 are identical. Deuel's applica-

tion does not describe the chemical structure of, or state how to isolate and purify, any DNA or cDNA molecule except the disclosed human placental and bovine uterine cDNAs, which are the subject of claims 5 and 7.

Claims 4-7 on appeal are all independent claims and read, in relevant part, as follows:

4. A purified and isolated DNA sequence consisting of a sequence encoding human heparin binding growth factor of 168 amino acids having the following amino acid sequence:

Met Gln Ala . . . [remainder of 168 amino acid sequence].

5. The purified and isolated cDNA of human heparin-binding growth factor having the following nucleotide sequence:

GTCAAAGGCA . . . [remainder of 961 nucleotide sequence].

6. A purified and isolated DNA sequence consisting of a sequence encoding bovine heparin binding growth factor of 168 amino acids having the following amino acid sequence:

Met Gln Thr . . . [remainder of 168 amino acid sequence].

7. The purified and isolated cDNA of bovine heparin-

binding growth factor having the following nucleotide sequence:

GAGTGGAGAG . . . [remainder of 1196 nucleotide [**8] sequence].

Claims 4 and 6 generically encompass *all* isolated/purified DNA sequences (natural and synthetic) encoding human and bovine HBGFs, despite the fact that Deuel's application does not describe the chemical structure of, or tell how to obtain, any DNA or cDNA except the two disclosed cDNA molecules. Because of the redundancy of the genetic code, claims 4 and 6 each encompass an enormous number of DNA molecules, including the isolated/purified chromosomal DNAs encoding the human and bovine proteins. Claims 5 and 7, on the other hand, are directed to the specifically disclosed cDNA molecules encoding human and bovine HBGFs, respectively.

During prosecution, the examiner rejected claims 4-7 under *35 U.S.C. § 103* as unpatentable over the combined teachings of Bohlen [3] [*1556] and Maniatis. [4] The Bohlen reference discloses a group of protein growth factors designated as heparin-binding brain mitogens ("HBBMs") useful in treating burns and promoting the formation, maintenance, and repair of tissue, particularly neural tissue. Bohlen isolated three such HBBMs from human and bovine brain tissue. These proteins have respective molecular weights of 15 kD, 16 kD, and 18 kD. Bohlen determined [**9] the first 19 amino acids of the proteins' N-terminal sequences, which were found to be identical for human and bovine HBBMs. [5] Bohlen teaches that HBBMs are brain-specific, and suggests that the proteins may be homologous between species. The reference provides no teachings concerning DNA or cDNA coding for HBBMs.

3 European Patent Application No. 0326075, naming Peter Bohlen as inventor, published August 2, 1989.

4 Maniatis et al., *Molecular Cloning: A Laboratory Manual*, "Screening Bacteriophage [lambda] Libraries for Specific DNA Sequences by Recombination in *Escherichia coli*," Cold Spring Harbor Laboratory, New York, 1982, pp. 353-361.

5 Bohlen's disclosed N-terminal sequence for human and bovine HBBMs is Gly-Lys-Lys-Glu-Lys-Pro-Glu-Lys-Lys-Val-Lys-Lys-Ser-Asp-Cys-Gly-Glu-Trp-Gln. This sequence matches the first 19 amino acids of Deuel's disclosed N-terminal sequence.

Maniatis describes a method of isolating DNAs or cDNAs by screening a DNA or cDNA library with a gene probe. The reference [**10] outlines a general technique for cloning a gene; it does not describe how to isolate a particular DNA or cDNA molecule. Maniatis does not discuss certain steps necessary to isolate a target cDNA, *e.g.*, selecting a tissue-specific cDNA library containing a target cDNA and designing an oligonucleotide probe that will hybridize with the target cDNA.

The examiner asserted that, given Bohlen's disclosure of a heparin-binding protein and its N-terminal sequence and Maniatis's gene cloning method, it would have been *prima facie* obvious to one of ordinary skill in the art at the time of the invention to clone a gene for HBGF. [6] According to the examiner, Bohlen's published N-terminal sequence would have motivated a person of ordinary skill in the art to clone such a gene because cloning the gene would allow recombinant production of HBGF, a useful protein. The examiner reasoned that a person of ordinary skill in the art could have designed a gene probe based on Bohlen's disclosed N-terminal sequence, then screened a DNA library in accordance with Maniatis's gene cloning method to isolate a gene encoding an HBGF. The examiner did not distinguish between claims 4 and 6 generically [**11] directed to all DNA

sequences encoding human and bovine HBGFs and claims 5 and 7 reciting particular cDNAs.

> 6 The examiner and the Board apparently used the term "gene" to refer both to natural (chromosomal) DNA and synthetic cDNA. We will use the several terms as appropriate.

In reply, Deuel argued, *inter alia*, that Bohlen teaches away from the claimed cDNA molecules because Bohlen suggests that HBBMs are brain-specific and, thus, a person of ordinary skill in the art would not have tried to isolate corresponding cDNA clones from human placental and bovine uterine cDNA libraries. The examiner made the rejection final, however, asserting that

> the starting materials are not relevant in this case, because it was well known in the art at the time the invention was made that proteins, especially the general class of heparin binding proteins, are highly homologous between species and tissue type. It would have been entirely obvious to attempt to isolate a known protein from different tissue types and [**12] even different species.

No prior art was cited to support the proposition that it would have been obvious to screen human placental and bovine uterine cDNA libraries for the claimed cDNA clones. Presumably, the examiner was relying on Bohlen's suggestion that HBBMs may be homologous between species, although the examiner did not explain how homology between species suggests homology between tissue types.

The Board affirmed the examiner's final rejection. In its opening remarks, the Board noted that it is "constantly advised by the [*1557] patent examiners, who are highly skilled in this art, that cloning procedures are routine in the art." According to the Board, "the examiners urge that when the sequence of a protein is placed into the public domain, the gene is also placed into the public domain because of the routine nature of cloning techniques." Addressing the rejection at issue, the Board determined that Bohlen's disclosure of the existence and isolation of HBBM, a functional protein, would also advise a person of ordinary skill in the art that a gene exists encoding HBBM. The Board found that a person of ordinary skill in the art would have been motivated to isolate such [**13] a gene because the protein has useful mitogenic properties, and isolating the gene for HBBM would permit large quantities of the protein to be produced for study and possible commercial use. Like the examiner, the Board asserted, without explanation, that HBBMs are the same as HBGFs and that the genes encoding these proteins are identical. The Board concluded that "the Bohlen reference would have suggested to those of ordinary skill in this art that they should make the gene, and the Maniatis reference would have taught a technique for 'making' the gene with a reasonable expectation of success." Responding to Deuel's argument that the claimed cDNA clones were isolated from human placental and bovine uterine cDNA libraries, whereas the combined teachings of Bohlen and Maniatis would only have suggested screening a brain tissue cDNA library, the Board stated that "the claims before us are directed to the product and not the method of isolation. Appellants have not shown that the claimed DNA was not present in and could not have been readily isolated from the brain tissue utilized by Bohlen." Deuel now appeals. [7]

> 7 Deuel is supported in its appeal by an *amicus curiae* brief submitted by the Biotechnology Industry Organization and the Bay Area Science Center. Amici urge that, contrary to controlling precedent, the PTO has unlawfully adopted a *per se* rule that a gene is *prima facie* obvious when at least part of the amino acid sequence of the protein encoded by the gene is known in the prior art.

[**14] DISCUSSION

[HN1] Obviousness is a question of law, which we review *de novo*, though factual findings underlying the Board's obviousness determination are reviewed for clear error. *In re Vaeck, 947 F.2d 488, 493, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991)*; *In re Woodruff, 919 F.2d 1575, 1577, 16 USPQ2d 1934, 1935 (Fed. Cir. 1990)*. [HN2] The examiner bears the burden of establishing a *prima facie* case of obviousness. *In re Rijckaert, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed. Cir. 1993)*; *In re Oetiker, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992)*. [HN3] Only if this burden is met does the burden of coming forward with rebuttal argument or evidence shift to the applicant. *Rijckaert, 9 F.3d at 1532, 28 USPQ2d at 1956*. [HN4] When the references cited by the examiner fail to establish a *prima facie* case of obviousness, the rejection is improper and will be overturned. *In re Fine, 837 F.2d 1071, 1074, 5 USPQ2d 1596, 1598 (Fed. Cir. 1988)*.

On appeal, Deuel challenges the Board's determination that the applied references establish a *prima facie* case of obviousness. In response, the PTO maintains that the claimed invention would have been *prima facie* obvious [**15] over the combined teachings of Bohlen and Maniatis. Thus, the appeal raises the important question whether the combination of a prior art reference teaching a method of gene cloning, together with a reference disclosing a partial amino acid sequence of a protein, may

render DNA and cDNA molecules encoding the protein *prima facie* obvious under *§ 103*.

Deuel argues that the PTO failed to follow the proper legal standard in determining that the claimed cDNA molecules would have been *prima facie* obvious despite the lack of structurally similar compounds in the prior art. Deuel argues that the PTO has not cited a reference teaching cDNA molecules, but instead has improperly rejected the claims based on the alleged obviousness of a method of making the molecules. We agree.

Because Deuel claims new chemical entities in structural terms, a *prima facie* case of unpatentability requires that the teachings of the prior art suggest *the claimed compounds* to a person of ordinary skill in the art. [*1558] [HN5] Normally a *prima facie* case of obviousness is based upon structural similarity, *i.e.*, an established structural relationship between a prior art compound and the claimed compound. [**16] Structural relationships may provide the requisite motivation or suggestion to modify known compounds to obtain new compounds. For example, a prior art compound may suggest its homologs because homologs often have similar properties and therefore chemists of ordinary skill would ordinarily contemplate making them to try to obtain compounds with improved properties. Similarly, a known compound may suggest its analogs or isomers, either geometric isomers (cis v. trans) or position isomers (*e.g.*, ortho v. para).

In all of these cases, however, the prior art teaches a specific, structurally-definable compound and the question becomes whether the prior art would have suggested making the specific molecular modifications necessary to achieve the claimed invention. *See In re Jones, 958 F.2d 347, 351, 21 USPQ2d 1941, 1944 (Fed. Cir. 1992)*; *In re Dillon, 919 F.2d 688, 692, 16 USPQ2d 1897, 1901 (Fed. Cir. 1990)* (en banc) [HN6] ("structural similarity between claimed and prior art subject matter, . . . where the prior art gives reason or motivation to make the claimed compositions, creates a *prima facie* case of obviousness"), *cert. denied, 500 U.S. 904, 114 L. Ed. 2d 77, 111 S. Ct. 1682 (1991)*; *In re Grabiak* [**17] , *769 F.2d 729, 731-32, 226 USPQ 870, 872 (Fed. Cir. 1985)* ("In the case before us there must be adequate support in the prior art for the [prior art] ester/[claimed] thioester change in structure, in order to complete the PTO's *prima facie* case and shift the burden of going forward to the applicant."); *In re Lalu, 747 F.2d 703, 705, 223 USPQ 1257, 1258 (Fed. Cir. 1984)* ("The prior art must provide one of ordinary skill in the art the motivation to make the proposed molecular modifications needed to arrive at the claimed compound.").

Here, the prior art does not disclose any relevant cDNA molecules, let alone close relatives of the specific, structurally-defined cDNA molecules of claims 5 and 7 that might render them obvious. Maniatis suggests an allegedly obvious process for trying to isolate cDNA molecules, but that, as we will indicate below, does not fill the gap regarding the subject matter of claims 5 and 7. Further, while the general idea of the claimed molecules, their function, and their general chemical nature may have been obvious from Bohlen's teachings, and the knowledge that some gene existed may have been clear, the precise cDNA molecules of claims 5 and [**18] 7 would not have been obvious over the Bohlen reference because Bohlen teaches proteins, not the claimed or closely related cDNA molecules. The redundancy of the genetic code precluded contemplation of or focus on the specific cDNA molecules of claims 5 and 7. Thus, one could not have conceived the subject matter of claims 5 and 7 based on the teachings in the cited prior art because, until the claimed molecules were actually isolated and purified, it would have been highly unlikely for one of ordinary skill in the art to contemplate what was ultimately obtained. What cannot be contemplated or conceived cannot be obvious.

The PTO's theory that one might have been motivated to try to do what Deuel in fact accomplished amounts to speculation and an impermissible hindsight reconstruction of the claimed invention. It also ignores the fact that claims 5 and 7 are limited to specific compounds, and any motivation that existed was a general one, to try to obtain a gene that was yet undefined and may have constituted many forms. A general motivation to search for some gene that exists does not necessarily make obvious a specifically-defined gene that is subsequently obtained as a result [**19] of that search. More is needed and it is not found here.

The genetic code relationship between proteins and nucleic acids does not overcome the deficiencies of the cited references. A prior art disclosure of the amino acid sequence of a protein does not necessarily render particular DNA molecules encoding the protein obvious because the redundancy of the genetic code permits one to hypothesize an enormous number of DNA sequences coding for the protein. No particular one of these DNAs can be obvious unless there is something in the prior art to lead to the particular DNA and indicate that it should be [*1559] prepared. We recently held in *In re Baird, 16 F.3d 380, 29 USPQ2d 1550 (Fed. Cir. 1994)*, that [HN7] a broad genus does not necessarily render obvious each compound within its scope. Similarly, knowledge of a protein does not give one a conception of a particular DNA encoding it. Thus, *a fortiori*, Bohlen's disclosure of the N-terminal portion of a protein, which the PTO urges is the same as HBGF, would not have suggested the particular cDNA molecules defined by claims 5 and 7. This is so even though one skilled in the art knew that some

DNA, albeit not in purified and isolated form, [**20] did exist. The compounds of claims 5 and 7 are specific compounds not suggested by the prior art. A different result might pertain, however, if there were prior art, *e.g.*, a protein of sufficiently small size and simplicity, so that lacking redundancy, each possible DNA would be obvious over the protein. *See In re Petering, 49 C.C.P.A. 993, 301 F.2d 676 (CCPA 1962)* (prior art reference disclosing limited genus of 20 compounds rendered every species within the genus unpatentable). That is not the case here.

The PTO's focus on known methods for potentially isolating the claimed DNA molecules is also misplaced because the claims at issue define compounds, not methods. *See In re Bell, 991 F.2d 781, 785, 26 USPQ2d 1529, 1532 (Fed. Cir. 1993)*. In *Bell*, the PTO asserted a rejection based upon the combination of a primary reference disclosing a protein (*and its complete amino acid sequence*) with a secondary reference describing a general method of gene cloning. We reversed the rejection, holding in part that "the PTO's focus on Bell's method is misplaced. Bell does not claim a method. Bell claims compositions, and the issue is the obviousness of the claimed compositions, not of the method [**21] by which they are made." *Id.*

We today reaffirm the principle, stated in Bell, that [HN8] the existence of a general method of isolating cDNA or DNA molecules is essentially irrelevant to the question whether the specific molecules themselves would have been obvious, in the absence of other prior art that suggests the claimed DNAs. A prior art disclosure of a process *reciting a particular compound* or obvious variant thereof as a product of the process is, of course, another matter, raising issues of anticipation under *35 U.S.C. § 102* as well as obviousness under *§ 103*. Moreover, where there is prior art that suggests a claimed compound, the existence, or lack thereof, of an enabling process for making that compound is surely a factor in any patentability determination. *See In re Brown, 51 C.C.P.A. 1254, 329 F.2d 1006, 141 USPQ 245 (CCPA 1964)* (reversing rejection for lack of an enabling method of making the claimed compound). There must, however, still be prior art that suggests the claimed compound in order for a *prima facie* case of obviousness to be made out; as we have already indicated, that prior art was lacking here with respect to claims 5 and 7. Thus, even if, as the examiner [**22] stated, the existence of general cloning techniques, coupled with knowledge of a protein's structure, might have provided motivation to prepare *a* cDNA or made it obvious to prepare *a* cDNA, that does not necessarily make obvious a particular claimed cDNA. "Obvious to try" has long been held not to constitute obviousness. *In re O'Farrell, 853 F.2d 894, 903, 7 USPQ2d 1673, 1680-81 (Fed. Cir. 1988)*. A general incentive does not make obvious a particular result, nor does the existence of techniques by which those efforts can be carried out. Thus, Maniatis's teachings, even in combination with Bohlen, fail to suggest the claimed invention.

The PTO argues that a compound may be defined by its process of preparation and therefore that a conceived process for making or isolating it provides a definition for it and can render it obvious. It cites *Amgen Inc. v. Chugai Pharmaceutical Co., 927 F.2d 1200, 18 USPQ2d 1016* (Fed. Cir.), *cert. denied, 502 U.S. 856 (1991)*, for that proposition. We disagree. [HN9] The fact that one can conceive a general process in advance for preparing an *undefined* compound does not mean that a claimed *specific* compound was precisely envisioned [**23] and therefore obvious. A substance may indeed be defined by its process of preparation. That occurs, however, when it has already been prepared by that process and one therefore knows that the result of that process is the stated compound. The process is part of the definition of the compound. [*1560] But that is not possible in advance, especially when the hypothetical process is only a general one. Thus, a conceived method of preparing some undefined DNA does not define it with the precision necessary to render it obvious over the protein it encodes. We did not state otherwise in *Amgen*. *See Amgen, 927 F.2d at 1206-9, 18 USPQ2d at 1021-23* (isolated/purified human gene held nonobvious; no conception of gene without envisioning its precise identity despite conception of general process of preparation).

We conclude that, because the applied references do not teach or suggest the claimed cDNA molecules, the final rejection of claims 5 and 7 must be reversed. *See also Bell, 991 F.2d at 784-85, 26 USPQ2d at 1531-32* (human DNA sequences encoding IGF proteins nonobvious over asserted combination of references showing gene cloning method and complete amino acid sequences of IGFs).

Claims [**24] 4 and 6 are of a different scope than claims 5 and 7. As is conceded by Deuel, they generically encompass all DNA sequences encoding human and bovine HBGFs. Written in such a result-oriented form, claims 4 and 6 are thus tantamount to the general idea of all genes encoding the protein, all solutions to the problem. Such an idea might have been obvious from the *complete* amino acid sequence of the protein, coupled with knowledge of the genetic code, because this information may have enabled a person of ordinary skill in the art to envision the idea of, and, perhaps with the aid of a computer, even identify all members of the claimed genus. The Bohlen reference, however, only discloses a partial amino acid sequence, and thus it appears that, based on the above analysis, the claimed genus would not have been obvious over this prior art disclosure. We

will therefore also reverse the final rejection of claims 4 and 6 because neither the Board nor the patent examiner articulated any separate reasons for holding these claims unpatentable apart from the grounds discussed above.

One further matter requires comment. Because Deuel's patent application does not describe how to obtain any DNA [**25] except the disclosed cDNA molecules, claims 4 and 6 may be considered to be inadequately supported by the disclosure of the application. *See generally Amgen Inc. v. Chugai Pharmaceutical Co., 927 F.2d 1200, 1212-14, 18 USPQ2d 1016, 1026-28* (Fed. Cir.) (generic DNA sequence claims held invalid under *35 U.S.C. § 112*, first paragraph), *cert. denied, 502 U.S. 856 (1991)*; *In re Fisher, 57 C.C.P.A. 1099, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970)* (*Section 112* "requires that the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art."). As this issue is not before us, however, we will not address whether claims 4 and 6 satisfy the enablement requirement of *§ 112*, first paragraph, but will leave to the PTO the question whether any further rejection is appropriate.

We have considered the PTO's remaining arguments and find them not persuasive.

CONCLUSION

The Board's decision affirming the final rejection of claims 4-7 is reversed.

REVERSED

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LEXSEE 958 F.2D 347

IN RE RITA S. JONES, MICHAEL T. CHIRCHIRILLO and JOHNNY L. BURNS

91-1380

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

958 F.2d 347; 1992 U.S. App. LEXIS 2752; 21 U.S.P.Q.2D (BNA) 1941

February 28, 1992, Decided

SUBSEQUENT HISTORY: As Corrected March 5, 1992.

PRIOR HISTORY: [**1] Appeal from: U.S. Patent & Trademark Office, Board of Patent Appeals & Interferences

DISPOSITION: REVERSED.

CASE SUMMARY:

PROCEDURAL POSTURE: Claimant inventors appealed from a decision of the Patent and Trademark Office Board of Patent Appeals and Interferences sustaining the rejection of an invention as unpatentable under *35 U.S.C.S. § 103*.

OVERVIEW: Claimant inventors filed a patent application for a novel salt. The Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences upheld the examiner's rejection of claimant's invention as unpatentable under *35 U.S.C.S. § 103* for obviousness. The PTO found that the claimed salt of dicamba and the salt of dicamba specifically disclosed by prior art were closely related in structure, and that based upon the expectation that compounds similar in structure would have similar properties, a prima facie case of obviousness had arisen. On appeal, the court reversed, holding that the PTO had not established a prima facie case of obviousness, and thus did not shift to claimants the burden of coming forward with evidence of non-obviousness. The court found that the two salts were not so closely related in structure as to render claimants' salt prima facie obvious in view of the prior art.

OUTCOME: The court reversed, concluding that the Patent and Trademark Office had not made a prima facie case of obviousness.

LexisNexis(R) Headnotes

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN1] Generalization is to be avoided insofar as specific structures are alleged to be prima facie obvious one from the other.

Patent Law > Nonobviousness > Elements & Tests > General Overview
Patent Law > Nonobviousness > Evidence & Procedure > Prima Facie Obviousness
[HN2] Every case, particularly those raising the issue of obviousness under *35 U.S.C.S. § 103*, must necessarily be decided upon its own facts.

Patent Law > Nonobviousness > Elements & Tests > Ordinary Skill Standard
Patent Law > Nonobviousness > Elements & Tests > Prior Art
Patent Law > Nonobviousness > Evidence & Procedure > Prima Facie Obviousness
[HN3] Before the Patent and Trademark Office may combine the disclosures of two or more prior art references in order to establish prima facie obviousness, there must be some suggestion for doing so, found either in the references themselves or in the knowledge generally available to one of ordinary skill in the art.

Patent Law > Claims & Specifications > Enablement Requirement > General Overview
Patent Law > Inequitable Conduct > General Overview
Patent Law > Nonobviousness > Evidence & Procedure > General Overview

[HN4] In order to make a prima facie case of obviousness, the prior art must provide one of ordinary skill in the art the motivation to make the proposed modifications needed to arrive at the claimed invention.

COUNSEL: Melvyn M. Kassenoff, Sandoz Corporation Patent & Trademark Dept., of East Hanover, New Jersey, argued for appellant. With him on the brief were Gerald D. Sharkin and Richard E. Vila. Also on the brief was Joanne M. Giesser, of Palo Alto, California.

Harris A. Pitlock, Associate Solicitor, of Arlington, Virginia, argued for appellee. With him on the brief was Fred E. McKelvey, Solicitor. Of counsel was Richard E. Schafer, Patent & Trademark Office.

JUDGES: Before RICH, ARCHER, and CLEVENGER, Circuit Judges.

OPINION BY: RICH

OPINION

[*348] RICH, *Circuit Judge.*

Rita S. Jones et al. (collectively Jones) appeal from the April 15, 1991 decision of the Patent and Trademark Office (PTO) Board of Patent Appeals and Interferences (Board), Appeal No. 90-1920, sustaining the rejection of claim 1, the only claim of application Ser. No. 07/099,279, titled "The 2-(2'-Aminoethoxy)Ethanol Salt of Dicamba," as unpatentable under *35 USC 103*. We conclude that the PTO has not presented a *prima facie* case of obviousness, and therefore *reverse*.

The Invention

The claimed invention is a novel salt of 2-methoxy-3,6-dichlorobenzoic acid, [**2] which acid is commonly referred to as "dicamba." A known herbicide, dicamba has typically been sold in the form of its known dimethylamine salt.

The sole claim of the application on appeal reads:

1. The 2-(2'-aminoethoxy)ethanol salt of dicamba.

The claimed salt has the following structure:

[SEE STRUCTURE ILLUSTRATION IN ORIGINAL]

[*349] *The Rejection*

Claim 1 stands rejected as obvious in view of the combined teachings of the following references:

Richter	U.S. Patent No. 3,013,054	Dec. 12, 1961
Moyle et al.	U.S. Patent No. 3,056,669	Oct. 2, 1962
Balassa	U.S. Patent No. 3,725,031	Apr. 3, 1973
Zorayan et al.	88 Chem. Abstracts No. 52300j	1978
Wideman	86 Chem. Abstracts No. 43711a	1977

Richter, which all agree is the closest prior art, discloses dicamba in free acid, ester, and salt forms, for use as a herbicide. Among the salt forms disclosed are substituted ammonium salts, a genus which admittedly encompasses the claimed salt. Richter does not specifically disclose the claimed 2-(2'-aminoethoxy)ethanol salt, however. Most notably, Richter discloses (emphasis and bracketed word ours):

Compositions in which X is substituted ammonium are amine salts of 2-methoxy-3,6-dichlorobenzoic [**3] acid [dicamba] and are prepared by the addition of the free acid to various amines. Typical amines which can be used to prepare such amine salts are dimethylamine, trimethylamine, triethylamine, diethanolamine, triethanolamine, isopropylamine, morpholine, and the like. *The resulting products are, respectively the dimethylamino, trimethylamino, triethylamino, diethanolamino, triethanolamino, isopropylamino, and morpholino salts of 2-methoxy-3,6-dichlorobenzoic acid.*

Zorayan teaches the amine (H[2]N(CH[2]CH[2]O)[2]H) used to make the claimed salt, as well as the use of that amine in the preparation of surfactants for shampoos, bath preparations, and emulsifiers.

Wideman also teaches the amine disclosed in Zorayan.

The content of the remaining references is unnecessary to our decision.

The Board upheld the examiner's rejection of claim 1 as obvious, finding that the claimed 2-(2'-aminoethoxy)ethanol salt of dicamba and the diethanolamine salt of dicamba specifically disclosed by Richter were "closely related in structure," and that based

upon the expectation that "compounds similar in structure will have similar properties," a *prima facie* case of obviousness had arisen. The Board [**4] found that Jones' rebuttal evidence (Rule 132 declarations and data reported in the specification) failed to "compare the claimed subject matter with the closest prior art," and accordingly did not serve to rebut the *prima facie* case. This appeal followed.

Analysis

The Solicitor contends that the claimed salt falls within the genus of substituted amine salts of dicamba disclosed by Richter, and that, like Richter's genus, the claimed compound has herbicidal activity. Thus, the Solicitor urges, under the circumstances of this case, (1) the genus/species relationship and (2) the common utility of the claimed and prior art compounds support the Board's holding of *prima facie* obviousness. Moreover, the Solicitor adds, although the claimed compound is neither a homolog nor a position isomer of those salts specifically disclosed in Richter, it is structurally similar thereto, particularly the diethanolamino salt noted by the Board.

The question of "structural similarity" in chemical patent cases has generated a body of patent law unto itself. [1] Particular types [*350] or categories of structural similarity without more have, in past cases, given rise to *prima facie* obviousness; [**5] *see, e.g., In re Dillon, 919 F.2d 688, 692-94, 16 USPQ2d 1897, 1900-02 (Fed. Cir. 1990)* (tri-orthoesters and tetra-orthoesters), *cert. denied, U.S. , 111 S. Ct. 1682 (1991)*; *In re May, 574 F.2d 1082, 197 USPQ 601 (CCPA 1978)* (stereoisomers); *In re Wilder, 563 F.2d 457, 195 USPQ 426 (CCPA 1977)* (adjacent homologs and structural isomers); *In re Hoch, 57 C.C.P.A. 1292, 428 F.2d 1341, 166 USPQ 406 (CCPA 1970)* (acid and ethyl ester). However, none of these types of structural similarity are involved here. And in any event, this court has previously stated that [HN1] generalization is to be avoided insofar as specific structures are alleged to be *prima facie* obvious one from the other. *In re Grabiak, 769 F.2d 729, 731, 226 USPQ 870, 872 (Fed. Cir. 1985).*

1 *See generally* Helmuth A. Wegner, "Prima Facie Obviousness of Chemical Compounds," 6 *Am. Pat. L. Assoc. Q. J.* 271 (1978).

[**6] On the basis of the record before us, we cannot sustain the Board's conclusion that the claimed salt and the diethanolamino salt disclosed by Richter are so "closely related in structure" as to render the former *prima facie* obvious in view of the latter. The claimed salt is a primary amine with an ether linkage. The diethanolamino salt disclosed by Richter is a secondary amine, without an ether linkage:

[SEE ILLUSTRATION IN ORIGINAL]

In addition, the only substituted ammonium salt of dicamba expressly disclosed by Richter having an ether linkage is the morpholino salt, which is *cyclic* in structure:

[SEE STRUCTURE ILLUSTRATION IN ORIGINAL]

The claimed salt is, plainly, acyclic; i.e., linear. Lastly, while the isopropylamino salt disclosed by Richter is a primary amine, as is the claimed salt, its isostructure is quite different:

[SEE STRUCTURE ILLUSTRATION IN ORIGINAL]

The lack of close similarity of structure is not negated by the fact that the claimed salt is a member of Richter's broadly disclosed genus of substituted ammonium salts of dicamba. The Solicitor contends that "the relative size of the genus disclosed by the prior art would not appear to be a controlling [**7] factor in determining whether a *prima facie* case of obviousness exists for a species encompassed within the described genus," citing *Merck & Co. v. Biocraft Labs., Inc., 874 F.2d 804, 806-09, 10 USPQ2d 1843, 1845-48* (Fed. Cir.), *cert. denied, 493 U.S. 975, 110 S. Ct. 498, 107 L. Ed. 2d 502, 110 S. Ct. 498 (1989)*. We decline to extract from *Merck* the rule that the Solicitor appears to suggest--that regardless of how broad, a disclosure of a chemical genus renders obvious any species that happens to fall within it. In *Merck*, at issue on appeal was whether claims to a composition of two diuretics, amiloride and hydrochlorothiazide, present in a particular "medically synergistic" weight ratio, would have been obvious in view of a specific prior art disclosure of amiloride in combination with hydrochlorothiazide, one of 1200 such combinations disclosed in the prior art reference. *Id. at 806, 10 USPQ2d at 1845*. Based on the facts before it, including evidence at trial that the experimentation needed to arrive at the claimed dosage was "nothing more than routine," *id. at 809, 10 USPQ2d at 1847*, [**8] the court held that the claimed invention would have been obvious. In contrast, though Richter discloses the potentially infinite genus of "substituted ammonium salts" of dicamba, and lists several such salts, the salt claimed here is not specifically disclosed. Nor, as we have explained above, is the claimed salt sufficiently similar in structure to those specifically disclosed in Richter as to render it *prima facie* obvious. [HN2] Every case, particularly those raising the issue of obviousness under *section 103*, must necessarily be decided upon its own facts.

[*351] The Solicitor points out that, given the breadth of forms of dicamba (free acid, ester, or salt) disclosed by Richter as having herbicidal utility, one of

ordinary skill in the art would appreciate that the dicamba group has significance with respect to imparting herbicidal activity to dicamba compounds. Thus, the Solicitor contends, one skilled in the art would have been motivated to use, with dicamba, substituted ammonium salts made from a known amine, such as the amine disclosed by Zorayan and Wideman, and would have expected such a salt to have herbicidal activity. [HN3] Before the PTO may combine the disclosures of two or more [**9] prior art references in order to establish *prima facie* obviousness, there must be some suggestion for doing so, found either in the references themselves or in the knowledge generally available to one of ordinary skill in the art. *In re Fine, 837 F.2d 1071, 1074, 5 USPQ2d 1596, 1598-99 (Fed. Cir. 1988).* We see no such suggestion in Zorayan, which is directed to shampoo additives, nor in Wideman, which teaches that the amine used to make the claimed compound is a byproduct of the production of morpholine. Nor does the broad disclosure of Richter fill the gap, for the reasons discussed above.

Conspicuously missing from this record is any *evidence,* other than the PTO's speculation (if it be called evidence) that one of ordinary skill in the herbicidal art would have been motivated to make the modifications of the prior art salts necessary to arrive at the claimed 2-(2'-aminoethoxy)ethanol salt. *See Grabiak, 769 F.2d at 731-32, 226 USPQ at 872* ("In the case before us there must be adequate support in the prior art for the [prior art] ester/[claimed] thioester change in structure, in order to complete the PTO's *prima* [**10] *facie* case and shift the burden of going forward to the applicant."); *In re Lalu, 747 F.2d 703, 705, 223 USPQ 1257, 1258 (Fed. Cir. 1984)* [HN4] ("The prior art must provide one of ordinary skill in the art the motivation to make the proposed molecular modifications needed to arrive at the claimed compound.")

Conclusion

We conclude that the PTO did not establish a *prima facie* case of obviousness, and thus did not shift to Jones the burden of coming forward with unexpected results or other objective evidence of non-obviousness. Accordingly, the decision of the Board is

REVERSED.

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U.S. Appln. No. 10/770,639
Reference No. 22

LEXSEE 919 F.2D 688

IN re DIANE M. DILLON

No. 88-1245

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

919 F.2d 688; 1990 U.S. App. LEXIS 19768; 16 U.S.P.Q.2D (BNA) 1897

November 9, 1990, Decided

PRIOR HISTORY: [**1] Appealed from: United States Patent & Trademark Office Board of Patent Appeals and Interferences.

CASE SUMMARY:

PROCEDURAL POSTURE: Petitioner appealed the judgment of the United States Patent and Trademark Office Board of Patent Appeals and Interferences rejecting certain claims in a patent application.

OVERVIEW: Petitioner's patent application described and claimed her discovery that the inclusion of certain tetra-orthoester compounds in hydrocarbon fuel would reduce the emission of solid particulates during combustion of fuel. The Board of Patent Appeals and Interferences rejected her patent application, holding all the claims to be unpatentable on the ground of obviousness under *35 U.S.C.S. § 103*. On appeal, the court held that properties had to be considered in the overall evaluation of obviousness and the lack of any disclosure of useful properties for a prior art compound might indicate a lack of motivation to make related compounds, thereby precluding a prima facie case. But, it was not correct that similarity of structure and a suggestion of the activity of an applicant's compounds in the prior art were necessary before a prima facie case was established.

OUTCOME: The court affirmed the judgment of the Board of Patent Appeals and Interferences rejecting certain claims in petitioner's patent application because petitioner failed to overcome the presumption of obviousness.

LexisNexis(R) Headnotes

Patent Law > Claims & Specifications > Claim Language > Representative Claims
Patent Law > U.S. Patent & Trademark Office Proceedings > Appeals
Patent Law > U.S. Patent & Trademark Office Proceedings > Interferences > Patentability & Priority Determinations
[HN1] It is not the practice of the appellate court to review claims that an applicant has not separately argued at the Board of Patent Appeals and Interferences level, because, inter alia, the appellate court lacks the benefit of the Board's reasoned decision on the separate patentability of those claims.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN2] A prima facie case of obviousness is not deemed made unless both (1) the new compound or composition is structurally similar to the reference compound or composition and (2) there is some suggestion or expectation in the prior art that the new compound or composition will have the same or a similar utility as that discovered by the applicant.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN3] Structural similarity between claimed and prior art subject matter, proved by combining references or otherwise, where the prior art gives reason or motivation to make the claimed compositions, creates a prima facie case of obviousness, and that the burden (and opportunity) then falls on an applicant to rebut that prima facie case. Such rebuttal or argument can consist of a comparison of test data showing that the claimed compositions possess unexpectedly improved properties or properties that the prior art does not have that the prior art is so deficient that there is no motivation to make what might

otherwise appear to be obvious changes or any other argument or presentation of evidence that is pertinent.

Patent Law > Inequitable Conduct > General Overview
Patent Law > Nonobviousness > Evidence & Procedure > Prima Facie Obviousness
Patent Law > Ownership > Patents as Property
[HN4] All evidence of the properties of claimed compositions and prior art must be considered in determining the ultimate question of patentability, but it is also clear that the discovery that a claimed composition possesses a property not disclosed for the prior art subject matter, does not by itself defeat a prima facie case. Each situation must be considered on its own facts, but it is not necessary in order to establish a prima facie case of obviousness that both a structural similarity between a claimed and prior art compound (or a key component of a composition) be shown and that there be a suggestion in or expectation from the prior art that the claimed compound or composition will have the same or a similar utility as one newly discovered by applicant. To the extent that *In re Wright, 848 F. 2d 1554*, suggests or holds to the contrary, it is overruled.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN5] The statement that a prima facie obviousness rejection is not supported if no reference shows or suggests the newly-discovered properties and results of a claimed structure is not the law.

Patent Law > Anticipation & Novelty > General Overview
Patent Law > Inequitable Conduct > Effect, Materiality & Scienter > General Overview
[HN6] The determination that a reference is from a nonanalogous art is two-fold. First, a court decides if the reference is within the field of the inventor's endeavor. If it is not, a court proceeds to determine whether the reference is reasonably pertinent to the particular problem with which the inventor was involved.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN7] Even substitution of an unobvious starting material into an old process does not necessarily result in an unobvious process.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN8] The established practice of rejecting closely-related compounds as prima facie obvious has been qualified by the rule that a presumption is not created when the reference compound is so lacking in any utility that there is no motivation to make close relatives.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN9] Properties are relevant to the creation of a prima facie case in the sense of affecting the motivation of a researcher to make compounds closely related to or suggested by a prior art compound, but it is not required, as stated in the dissent, that the prior art disclose or suggest the properties newly-discovered by an applicant in order for there to be a prima facie case of obviousness.

COUNSEL: James H. Laughlin, Jr., Benoit, Smith & Laughlin, of Arlington, Virginia, argued for Appellant. With him on the brief was Gregory F. Wirzbicki, Unocal Corporation, Brea, California, of Counsel.

Fred E. McKelvey, Solicitor, Office of the Solicitor, of Arlington, Virginia, argued for Appellee. With him on the brief were Richard E. Schafer, Associate Solicitor and Joseph F. Nakamura.

Allen S. Melser and Linda J. Shapiro, Mason, Fenwick & Lawrence, of Washington, District of Colubmia, were on the brief for Amicus Curiae Chemical Manufacturers Association.

William S. Thompson, President, American Intellectual Property Law Association, of Arlington, Virginia, was on the brief for Amicus Curiae American Intellectual Property Law Association. With him on the brief were Donald R. Dunner, Herbert H. Mintz, and Thomas L. Irving, Finnegan, Henderson, Farabow, Garrett & Dunner, of Washington, District of Columbia Also on the brief was Harold C. Wegner, Wegner & Bretschneider, of Washington, District of Columbia, of Counsel.

Professor Irving Kayton, of Washington, District of Columbia, [**2] was on the brief for Amicus Curiae Professor Irving Kayton.

Robert A. Chittum, President, Rochester Patent Law Association, of Rochester, New York, was on the brief for Amicus Curiae Rochester Patent Law Association.

JUDGES: Nies, Chief Judge, Rich, Circuit Judge, Cowen, Senior Circuit Judge, Markey, Newman, Archer, Mayer, Michel, Plager, Lourie, Clevenger, and Rader, Circuit Judges. Archer, Circuit Judge, with whom Markey and Michel, Circuit Judges, join, joining-

in-part. Newman, Circuit Judge, with whom Cowen, Senior Circuit Judge, and Mayer, Circuit Judge, join, dissenting.

* Circuit Judge Markey vacated the position of Chief Judge on June 27, 1990.

OPINION BY: LOURIE

OPINION

[*690] LOURIE, Circuit Judge

Diane M. Dillon, assignor to Union Oil Company of California, appeals the November 25, 1987, decision of the Board of Patent Appeals and Interferences (Board) of the United States Patent and Trademark Office (PTO), Appeal No. 87-0944, rejecting claims 2-14, 16-22, and 24-37, all the remaining claims of patent application Serial No. 671,570 entitled "Hydrocarbon Fuel Composition." We affirm the rejection of all of the claims. [1]

1 A panel of this court heard this appeal and reversed the Board on December 29, 1989. *892 F.2d 1554, 13 USPQ2d 1337*. The PTO petitioned for rehearing and suggested rehearing in banc on February 12, 1990. Rehearing in banc was ordered on May 21, 1990, and the judgment which was entered on December 29, 1989, was vacated, the accompanying opinion being withdrawn.

[**3] *The Invention*

Dillon's patent application describes and claims her discovery that the inclusion of certain tetra-orthoester compounds in hydrocarbon fuel compositions will reduce the emission of solid particulates (*i.e.*, soot) during combustion of the fuel. In this appeal Dillon asserts the patentability of claims to hydrocarbon fuel compositions containing these tetra-orthoesters, and to the method of reducing particulate emissions during combustion by combining these esters with the fuel before combustion.

Claim 2 is the broadest composition claim:

> 2. A composition comprising: a hydrocarbon fuel; and a sufficient amount of at least one orthoester so as to reduce the particulate emissions from the combustion of the hydrocarbon fuel, wherein the orthoester is of the formula:
>
> [SEE ILLUSTRATION IN ORIGINAL]
>
> wherein R[5], R[6], R[7], and R[8], are the same or different monovalent organic radical comprising 1 to about 20 carbon atoms.

The broadest method claim is claim 24:

> 24. A method of reducing the particulate emissions from the combustion of a hydrocarbon fuel comprising combusting a mixture of the hydrocarbon fuel and a sufficient [**4] amount of at least [*691] one orthoester so as to reduce the particulate emissions, wherein the orthoester is of the formula:
>
> [SEE ILLUSTRATION IN ORIGINAL]
>
> wherein R[5], R[6], R[7], and R[8], are the same or different monovalent organic radical comprising 1 to about 20 carbon atoms.

The other claims contain additional limitations and thus are narrower in scope.

The tetra-orthoesters are a known class of chemical compounds. It is undisputed that their combination with hydrocarbon fuel, for any purpose, is not shown in the prior art, and that their use to reduce particulate emissions from combustion of hydrocarbon fuel is not shown or suggested in the prior art.

The Rejection

The Board held all of the claims to be unpatentable on the ground of obviousness, *35 U.S.C. § 103*, in view of certain primary and secondary references. As primary references the Board relied on two Sweeney U.S. patents, 4,390,417 ('417) and 4,395,267 ('267). Sweeney '417 describes hydrocarbon fuel compositions containing specified chemical compounds, *viz.*, ketals, acetals, and tri-orthoesters, [2] used for "dewatering" the fuels, particularly [**5] diesel oil. Sweeney '267 describes three-component compositions of hydrocarbon fuels heavier than gasoline, immiscible alcohols, and tri-orthoesters, wherein the tri-orthoesters serve as cosolvents to prevent phase separation between fuel and alcohol. The Board explicitly found that the Sweeney patents do not teach the use of the tetra-orthoesters recited in appellant's claims.

2 Tri-orthoesters have three -OR groups bonded to a central carbon atom, and the fourth carbon bond is to hydrogen or a hydrocarbon group (-R); they are represented as C(R)(OR)[3]. Tetra-

orthoesters have four -OR groups bonded to a central carbon atom, and are represented as C(OR)[4]; see Dillon's claims, *supra*.

The Board cited Elliott U.S. Patent 3,903,006 and certain other patents, including Howk U.S. Patent 2,840,613, as secondary references. Elliott describes tri-orthoesters and tetra-orthoesters for use as water scavengers in hydraulic (non-hydrocarbon) fluids. The Board stated that the Elliott reference shows equivalence [**6] between tetra-orthoesters and tri-orthoesters, and that "it is clear from the combined teachings of these references. . . that [Dillon's tetra-orthoesters] would operate to remove water from non-aqueous liquids by the same mechanism as the orthoesters of Sweeney."

The Board stated that there was a "reasonable expectation" that the tri- and tetra-orthoester fuel compositions would have similar properties, based on "close structural and chemical similarity" between the tri- and tetra-orthoesters and the fact that both the prior art and Dillon use these compounds as "fuel additives." The Commissioner argues on appeal that the claimed compositions and method "would have been *prima facie* obvious from combined teachings of the references." On this reasoning, the Board held that unless Dillon showed some unexpected advantage or superiority of her claimed tetra-orthoester fuel compositions as compared with tri-orthoester fuel compositions, Dillon's new compositions as well as her claimed method of reducing particulate emissions are unpatentable for obviousness. It found that no such showing was made.

The Issue

The issue before this court is whether the Board erred in rejecting [**7] as obvious under *35 U.S.C. § 103* claims to Dillon's new compositions and to the new method of reducing particulate emissions, when the additives in the new compositions are structurally similar to additives in known compositions, having a different use, but the new method of reducing particulate emissions is neither taught nor suggested by the prior art.

[*692] *The Broad Composition Claims*

Claim 2, the broadest composition claim, comprises a hydrocarbon fuel and an amount of tetra-orthoester sufficient to reduce the particulate emissions from the combustion of the hydrocarbon fuel. The other composition claims contain various limitations including a minimum amount of emission reduction to be achieved (claim 3), percentages of ester in the fuel (claims 4, 5, 16, 20, 21), use of different esters (claims 6-10, 17-19), use of different fuels (claims 11-14, 22), and the requirement that the composition be essentially free of alcohol (claims 36, 37).

The Board stated in its opinion that, to the extent that appellant had not chosen to separately argue the patentability of the appealed claims, "they stand or fall together," citing *In re Kaslow, 707 F.2d 1366, 1376, 217 U.S.P.Q. (BNA) 1089, 1096 (Fed. Cir. 1983).* [**8] We agree with the Board except as noted briefly below. [HN1] It is not the practice of this court to review claims that an applicant has not separately argued at the Board level, because, *inter alia*, we lack the benefit of the Board's reasoned decision on the separate patentability of those claims. *Id. at 1376, 217 U.S.P.Q. (BNA) at 1096*; *In re Herbert, 59 C.C.P.A. 1091, 461 F.2d 1390, 1391, 174 U.S.P.Q. (BNA) 259, 260 (CCPA 1972).* We will therefore review only the merits of the composition claims, as did the Board.

The Board found that the claims to compositions of a hydrocarbon fuel and a tetra-orthoester were *prima facie* obvious over Sweeney '417 and '267 in view of Elliott and Howk. We agree. Appellant argues that none of these references discloses or suggests the new use which she has discovered. That is, of course, true, but the composition claims are not limited to this new use; *i.e.*, they are not physically or structurally distinguishable over the prior art compositions except with respect to the orthoester component. We believe that the [**9] PTO has established, through its combination of references, that there is a sufficiently close relationship between the tri-orthoesters and tetra-orthoesters (see the cited Elliott and Howk references) in the fuel oil art to create an expectation that hydrocarbon fuel compositions containing the tetra-esters would have similar properties, including water scavenging, to like compositions containing the tri-esters, and to provide the motivation to make such new compositions. Howk teaches use of both tri- and tetra-orthoesters in a similar type of chemical reaction. Elliott teaches their equivalence for a particular practical use.

Our case law well establishes that such a fact situation gives rise to a *prima facie* case of obviousness. *See In re Shetty, 566 F.2d 81, 85, 195 USPQ 753, 755-56 (CCPA 1977)*; *In re Albrecht, 514 F.2d 1385, 1388, 185 USPQ 590, 593 (CCPA 1975)*; *In re Murch, 59 C.C.P.A. 1277, 464 F.2d 1051, 1054, 175 U.S.P.Q. (BNA) 89, 91 (CCPA 1972)*; *In re Hoch, 57 C.C.P.A. 1292, 428 F.2d 1341, 1343, 166 U.S.P.Q. (BNA) 406, 409 (CCPA 1970).*

Appellant cites *In re Wright, 848 F.2d 1216, 1219, 6 USPQ2d 1959, 1961 (Fed. Cir. 1988)*, [**10] for the proposition that a *prima facie* case of obviousness requires that the prior art suggest the claimed compositions' properties and the problem the applicant attempts to solve. The earlier panel opinion in this case, *In re Dillon, 892 F.2d 1554, 13 USPQ2d 1337* (now withdrawn), in fact stated "a [HN2] *prima facie* case of obviousness is not deemed made unless both (1) the new compound or composition is structurally similar to the reference com-

pound or composition and (2) there is some suggestion or expectation *in the prior art* that the new compound or composition will have the *same or a similar utility as that discovered by the applicant.*" *Id. at 1560, 13 USPQ2d at 1341* (emphasis added).

This court, in reconsidering this case *in banc*, reaffirms that [HN3] structural similarity between claimed and prior art subject matter, proved by combining references or otherwise, where the prior art gives reason or motivation to make the claimed compositions, creates [**11] a *prima facie* case of obviousness, and that the burden (and opportunity) then falls on an applicant to rebut that *prima facie* case. Such rebuttal or argument can consist of a comparison of test data showing that the claimed compositions possess unexpectedly improved properties or properties that the prior art does [*693] not have (*In re Albrecht, 514 F.2d 1389, 1396, 185 USPQ 585, 590 (CCPA 1975)*; *Murch, 464 F.2d at 1056, 175 USPQ at 92*), that the prior art is so deficient that there is no motivation to make what might otherwise appear to be obvious changes (*Albrecht, 514 F.2d at 1396, 185 USPQ at 590*; *In re Stemniski, 58 C.C.P.A. 1410, 444 F.2d 581, 170 USPQ 343 (CCPA 1971)*; *In re Ruschig, 52 C.C.P.A. 1238, 343 F.2d 965, 145 USPQ 274 (CCPA 1965))*, or any other argument or presentation of evidence that is pertinent. [HN4] There is no question that all evidence of the properties of the claimed compositions and the prior art must be considered in determining the ultimate question of patentability, [**12] but it is also clear that the discovery that a claimed composition possesses a property not disclosed for the prior art subject matter, does not by itself defeat a *prima facie* case. *Shetty, 566 F.2d at 86, 195 USPQ at 756.* Each situation must be considered on its own facts, but it is not necessary in order to establish a *prima facie* case of obviousness that both a structural similarity between a claimed and prior art compound (or a key component of a composition) be shown and that there be a suggestion in or expectation from *the prior art* that the claimed compound or composition will have the same or a similar utility *as one newly discovered by applicant.* To the extent that *Wright* suggests or holds to the contrary, it is hereby overruled. [HN5] In particular, the statement that a *prima facie* obviousness rejection is not supported if no reference shows or suggests the newly-discovered properties and results of a claimed structure is not the law. [3]

3 The earlier, now-withdrawn Dillon opinion, this opinion, and the dissent cite and rely on cases involving claims to chemical compounds, whereas this case involves compositions. The reason for this reliance is that, in this case, the principal difference between the claimed and prior art compositions is the difference between chemical compounds, *viz.*, tri-orthoesters and tetra-orthoesters. Cases dealing with chemical compounds are therefore directly analogous here and, in view of the history of this case and its *in banc* status, we will make much comment on these cases in this opinion. We do not, however, intend to imply that in all cases involving claimed compositions, structural obviousness between involved chemical compounds necessarily makes the claimed compositions *prima facie* obvious.

[**13] Under the facts we have here, as described above, we have concluded that a *prima facie* case has been established. The art provided the motivation to make the claimed compositions in the expectation that they would have similar properties. Appellant had the opportunity to rebut the *prima facie* case. She did not present any showing of data to the effect that her compositions had properties not possessed by the prior art compositions or that they possessed them to an unexpectedly greater degree. She attempted to refute the significance of the teachings of the prior art references. She did not succeed and we do not believe the PTO was in error in its decision.

Appellant points out that none of the references relates to the problem she confronted, citing *In re Wright*, and that the combination of references is based on hindsight. It is clear, however, that appellant's claims have to be considered as she has drafted them, *i.e.*, as compositions consisting of a fuel and a tetra-orthoester, and that Sweeney '417 and '267 describe the combination of a liquid fuel with a related compound, a tri-orthoester. While Sweeney does not suggest appellant's use, her composition claims [**14] are not limited to that use; [4] the claims merely recite compositions analogous to those in the Sweeney patents, and appellant has made no showing overcoming the *prima facie* presumption of similar properties for those analogous compositions. The mention in the appealed claims that the amount of orthoester must be sufficient to reduce particulate emissions is not a distinguishing limitation of the claims, unless that amount is different [*694] from the prior art and critical to the use of the claimed composition. *See In re Reni, 57 C.C.P.A. 857, 419 F.2d 922, 925, 164 USPQ 245, 247 (CCPA 1970).* That is not the case here. The amount of ester recited in the dependent claims can be from 0.05-49%, a very broad range; a preferred range is.05-9%, compared with a percentage in Sweeney '417 approximately equimolar to the amounts of water in the fuel which the ester is intended to remove (.01-5%).

4 The dissent misinterprets this comment as indicating that claims to new compounds and compositions must contain a limitation to a specific use, and states that past cases have rejected this proposition. Our comment only points out that

> the composition claims on appeal are not structurally or physically distinguishable from the prior art compositions by virtue of the recitation of their newly-discovered use.

[**15] Appellant attacks the Elliott patent as non-analogous art, being in the field of hydraulic fluids rather than fuel combustion. We agree with the PTO that the field of relevant prior art need not be drawn so narrowly. As this court stated in *In re Deminski, 796 F.2d 436, 442, 230 USPQ 313, 315 (Fed. Cir. 1986)* (quoting *In re Wood, 599 F.2d 1032, 1036, 202 USPQ 171, 174 (CCPA 1979))*:

> [HN6] the determination that a reference is from a nonanalogous art is therefore two-fold. First, we decide if the reference is within the field of the inventor's endeavor. If it is not, we proceed to determine whether the reference is reasonably pertinent to the particular problem with which the inventor was involved.

Following that test, one concerned with the field of fuel oils clearly is chargeable with knowledge of Sweeney '417, which discloses fuel compositions with tri-orthoesters for dewatering purposes, and chargeable with knowledge of other references to tri-orthoesters, including for use as dewatering agents for fluids, albeit other fluids. These references [**16] are "within the field of the inventor's endeavor." Moreover, the statement of equivalency between tri- and tetra-orthoesters in Elliott is not challenged. We therefore conclude that Elliott is not excludable from consideration as non-analogous art. It is evidence that supports the Board's holding that the prior art makes the claimed compositions obvious, a conclusion that appellant did not overcome.

Appellant urges that the Board erred in not considering the unexpected results produced by her invention and in not considering the claimed invention as a whole. The Board found, on the other hand, that no showing was made of unexpected results for the claimed compositions compared with the compositions of Sweeney. We agree. Clearly, in determining patentability the Board was obligated to consider all the evidence of the properties of the claimed invention as a whole, compared with those of the prior art. However, after the PTO made a showing that the prior art compositions suggested the claimed compositions, the burden was on the applicant to overcome the presumption of obviousness that was created, and that was not done. For example, she produced no evidence that her compositions possessed [**17] properties not possessed by the prior art compositions. Nor did she show that the prior art compositions and use were so lacking in significance that there was no motivation for others to make obvious variants. There was no attempt to argue the relative importance of the claimed compositions compared with the prior art. *See In re May, 574 F.2d 1082, 1092-95, 197 USPQ 601, 609-11 (CCPA 1978).*

Appellant's patent application in fact included data showing that the prior art compositions containing tri-orthoesters had equivalent activity in reducing particulate emissions (she apparently was once claiming such compositions with either tri-orthoesters or tetra-orthoesters). She asserts that the examiner used her own showing of equivalence against her in violation of the rule of *In re Ruff, 45 C.C.P.A. 1037, 256 F.2d 590, 596, 118 USPQ 340, 346 (CCPA 1958)*. While we caution against such a practice, it is clear to us that references by the PTO to the comparative data in the patent application were not employed as evidence of equivalence between the tri- and tetra-orthoesters; the PTO was simply pointing out that the applicant did not or apparently could [**18] not make a showing of superiority for the claimed tetra-ester compositions over the prior art tri-ester compositions.

Other Claims

As indicated above, the Board held that it would not consider the appealed claims separately, [*695] since appellant did not. Nonetheless, appellant, in her brief before the Board, did refer generally to claims directed to fuels essentially free of alcohol (Brief at 32, J. App. at 122), and the Board, in its decision, did respond, stating that no objective evidence was presented to establish any different or unexpected results from limiting the amount of alcohol to less than 1%, *In re Dillon*, No. 87-0944, slip op. at 9-10 (Bd. Pat. App. & Int. Nov. 25, 1987). We agree. Moreover, Sweeney '417 describes compositions which also contain less than 1% alcohol, so there is no real distinction in the claims over the prior art.

Appellant also argued in her brief to the Board that she "must contest" the examiner's position concerning claims limited to no more than 5 or 9 volume percent added orthoester, mentioning claims 16-21 and 27-31. The Board did not expressly deal with these claims, but it is clear to us that, since these limits do not result [**19] in an amount of ester different from that disclosed to remove water in Sweeney '417, and no criticality is shown for these limitations, the subject matter of these claims has not been shown to have been nonobvious.

Regarding the method claims, the Commissioner urges affirmance, citing *In re Durden, 763 F.2d 1406, 226 USPQ 359 (Fed. Cir. 1985)*, for the proposition that [HN7] even "substitution of an unobvious starting material into an old process does not necessarily result in an unobvious process." The PTO has, as the Commissioner urges here, applied *Durden* regularly to claims to proc-

esses of making and processes of using, on the ground that the type of step involved in the claimed process is not novel. [5]

5 *See* M. A. Litman, *Obvious Process Rejections Under 35 USC 103*, 71 JPTOS 775 (1989); H. C. Wegner, *Much Ado About Durden*, 71 JPTOS 785 (1989).

We make no judgment [**20] as to the patentability of claims that Dillon might have made and properly argued to a method directed to the novel aspects of her invention, except to question the lack of logic in a claim to a method of reducing particulate emissions by combusting. Suffice it to say that we do not regard *Durden* as authority to reject as obvious every method claim reading on an old *type of process*, such as mixing, reacting, reducing, etc. The materials used in a claimed process as well as the result obtained therefrom, must be considered along with the specific nature of the process, and the fact that new or old, obvious or nonobvious, materials are used or result from the process are only factors to be considered, rather than conclusive indicators of the obviousness or nonobviousness of a claimed process. When any applicant properly presents and argues suitable method claims, they should be examined in light of all these relevant factors, free from any presumed controlling effect of *Durden*. *Durden* did not hold that all methods involving old process steps are obvious; the court in that case concluded that the particularly claimed process was obvious; it refused to adopt an unvarying [**21] rule that the fact that nonobvious starting materials and nonobvious products are involved *ipso facto* makes the process nonobvious. Such an invariant rule always leading to the opposite conclusion is also not the law. Thus, we reject the Commissioner's argument that we affirm the rejection of the method claims under the precedent of *Durden*.

However, appellant did not argue in her brief the separate patentability of her method claims. The statement in her brief to the Board that "the invention 'as a whole' includes the property of the claimed compositions -- which property is taken advantage of in the method claims" (Brief at 22, J. App. at 112) is not such a separate argument, since it implies more an added argument for the patentability of the composition claims than an argument that, even if the claimed compositions are found to have been obvious, the claimed methods were nonobvious for particularly stated reasons. Moreover, no such reasons were particularly stated by Dillon. We will therefore not analyze these claims separately and affirm the Board's rejection on that basis.

[*696] *The Dissent*

The strong assertions by the dissent and its treatment of some [**22] of the case law impel us to make the following comments:

The dissent argues that our decision is "contrary to the weight and direction of . . . precedent, as embodied in over three decades of decision"; that we are resurrecting the "Hass-Henze" Doctrine, which was "discarded thirty years ago"; and that our holding today "changes what must be proved in order to patent a new chemical compound and composition and its new use, and thus changes what is patentable." We have done none of the above.

What we have done is to decide the case before us on the basis of long-established principles which had provided a stable understanding of the chemical patent law until the issuance of the original panel opinion in this case which we have now vacated. Our intent is to restore the law to its state existing before that panel opinion.

The length of the dissent and the number of cases it discusses may convey the impression that the weight of past jurisprudence is contrary to our holding today. That is not the case. The cited cases are simply not controlling on the facts of the present case or they are not contrary. Many do not deal with the requirements of a *prima facie* case. Some involve [**23] process claims, not compound or composition claims. Others are not pertinent for other reasons.

In brief, the cases establish that if an examiner considers that he has found prior art close enough to the claimed invention to give one skilled in the relevant chemical art the motivation to make close relatives (homologs, analogs, isomers, etc.) of the prior art compound(s), then there arises what has been called a presumption of obviousness or a *prima facie* case of obviousness. *In re Henze, 37 C.C.P.A. 1009, 181 F.2d 196, 85 USPQ 261 (CCPA 1950)*; *In re Hass, 31 C.C.P.A. 895, 141 F.2d 122, 127, 130, 60 USPQ 544, 548, 552 (CCPA 1944)*. The burden then shifts to the applicant, who then can present arguments and/or data to show that what appears to be obvious, is not in fact that, when the invention is looked at as a whole. *In re Papesch, 50 C.C.P.A. 1084, 315 F.2d 381, 137 USPQ 43 (CCPA 1963)*. The cases of *Hass* and *Henze* established the rule that, unless an applicant showed that the prior art compound lacked the property or advantage asserted for the claimed compound, the presumption of unpatentability was not overcome.

[**24] Exactly what facts constituted a *prima facie* case varied from case to case, but it was not the law that, where an applicant asserted that an invention possessed properties not known to be possessed by the prior art, no *prima facie* case was established unless the reference also showed the novel activity. There are cases, cited in

the dissent, in which a *prima facie* case was not established based on lack of structural similarity. *See In re Grabiak, 769 F.2d 729, 732, 226 USPQ 870, 872 (Fed. Cir. 1985)*; *In re Taborsky, 502 F.2d 775, 780-81, 183 USPQ 50, 55 (CCPA 1974)*. Some of the cited cases also contained language suggesting that the fact that the claimed and the prior art compounds possessed the same activity were added factors in the establishment of the *prima facie* case. *E.g.*, *In re Zeidler, 682 F.2d 961, 966, 215 USPQ 490, 494 (CCPA 1982)*; *In re Grunwell, 609 F.2d 486, 491, 203 USPQ 1055, 1058 (CCPA 1979)*; *In re Payne, 606 F.2d 303, 314, 203 USPQ 245, 255 (CCPA 1979)*; *In re Swan Wood, 582 F.2d 638, 641, 199 USPQ 137, 139 (CCPA 1978)*; *In re Lamberti, 545 F.2d 747, 751, 192 USPQ 278, 281 (CCPA 1976)*; [**25] *In re Susi, 58 C.C.P.A. 1074, 440 F.2d 442, 445, 169 USPQ 423, 426 (CCPA 1971)*. Those cases did not say, however, as the dissent asserts, that, in the absence of the similarity of activities, there would have been no *prima facie* case.

For example, the dissent quotes a statement in *Grabiak* that "when chemical compounds have 'very close' structural similarities and similar utilities, without more a *prima facie* case may be made." *769 F.2d at 731, 226 USPQ at 871*. That case does not state, as implied by the dissent, that without the similarity of utilities, there would not have been a *prima facie* case. A conclusion based on one set of facts does [*697] not necessarily rule out a similar conclusion with slightly different facts.

One case cited by the dissent as "rejecting the PTO's interpretation of *Henze* as establishing a 'legal presumption' of obviousness" was *In re Mills, 47 C.C.P.A. 1185, 281 F.2d 218, 126 USPQ 513 (CCPA 1960)*. All that case decided, however, was that a one-carbon member of a homologous series is too far from the prior art disclosure of 8 to 12 members to justify the presumption. *Id.* [**26] *at 223-24, 126 USPQ at 517-18*.

We will not review all the cases cited in the dissent, but *Stemniski* is an important case, for it overruled *Henze* and *In re Riden, 50 C.C.P.A. 1411, 318 F.2d 761, 138 USPQ 112 (CCPA 1963)* (a case similar to *Henze*), "to the extent that [they] are inconsistent with the views expressed herein." *444 F.2d at 587, 170 USPQ at 348*. The views that were expressed therein were that:

> where the prior art reference neither discloses nor suggests a utility for certain described compounds, why should it be said that a reference makes obvious to one of ordinary skill in the art an isomer, homolog or analog or related structure, when that mythical, but intensely practical, person knows of no "practical" reason to make the reference compounds, much less any structurally related compounds?

Id. at 586, 170 USPQ at 347. Thus, *Stemniski*, [HN8] rather than destroying the established practice of rejecting closely-related compounds as *prima facie* obvious, qualified it by holding that a presumption is not created [**27] when the reference compound is so lacking in any utility that there is no motivation to make close relatives.

Albrecht followed this line of thinking when it held that the prior art compound so irritated the skin that it could not be regarded as useful and therefore did not create a motivation to make related compounds. *514 F.2d at 1392, 1395-96, 185 USPQ at 587, 590*.

[HN9] Properties, therefore, *are* relevant to the creation of a *prima facie* case in the sense of affecting the motivation of a researcher to make compounds closely related to or suggested by a prior art compound, but it is not required, as stated in the dissent, that the prior art disclose or suggest the properties newly-discovered by an applicant in order for there to be a *prima facie* case of obviousness.

The dissent cites the seminal case of *Papesch*, suggesting that it rejected the principle that we now "adopt," thereby implying that we are weakening *Papesch*. We are doing nothing of the sort. *Papesch* indeed stated that a compound and all of its properties are inseparable and must be considered [**28] in the determination of obviousness. We heartily agree and intend not to retreat from *Papesch* one inch. *Papesch*, however, did not deal with the requirements for establishing a *prima facie* case, but whether the examiner had to consider the properties of an invention at all, when there was a presumption of obviousness. *315 F.2d at 391, 137 USPQ at 51*. The reference disclosed a lower homolog of the claimed compounds, so it was clear that impliedly a *prima facie* case existed; the question was whether, under those circumstances, the biological data were admissible at all. The court ruled that they were, *id. at 391, 137 USPQ at 51*, and we agree with that result. The dissent quotes the brief passage at the end of the *Papesch* opinion to the effect that the prior art must "at least to a degree" disclose the applicant's desired property, *id. at 392, 137 USPQ at 52*, but this brief mention was not central to the decision in that case and did not refer to the requirements of a *prima facie* case. *Papesch* is irrelevant to the question of the requirements for a *prima facie* case, which is the question we have here.

The dissent refers to a number [**29] of cases, including *In re Lunsford, 53 C.C.P.A. 986, 357 F.2d 380, 148 USPQ 716 (CCPA 1966)*, stating that the court had rejected the Patent Office's position of "structures only." That case must be understood in the context of the refusal of the examiner to consider any showing of improved properties, not in the context of a discussion whether a *prima facie* case was created. The compounds were conceded to be sufficiently close to the prior art that, without a showing [*698] of a significant difference in properties, they would have been obvious. *Id. at 381, 148 USPQ at 717.*

The dissent asserts that the *Shetty* case "diverged from the weight of [the court's] precedent" in holding that a *prima facie* case was made based on similarities of structure. The court, in that opinion, accepted the *prima facie* case and concluded that "appellant had offered no evidence of unobviousness, as by showing an actual difference in properties." *566 F.2d at 86, 195 USPQ at 756* (citing *Hoch*). The opinion does not suggest that the court was diverging from the weight of precedent.

The dissent mentions positions advanced by the Commissioner, [**30] including citing the *In re Mod, 56 C.C.P.A. 1041, 408 F.2d 1055, 161 USPQ 281 (CCPA 1969)* and *In re de Montmollin, 52 C.C.P.A. 1287, 344 F.2d 976, 145 USPQ 416 (CCPA 1965)* decisions. We do not, however, in today's decision necessarily adopt any positions of the Commissioner other than those stated in our opinion and note that neither *Mod* nor *de Montmollin* dealt with the requirements of a *prima facie* case. They concerned the question whether the existence of a new property for claimed compounds in addition to a property common to both the claimed and related prior art compounds rendered the claimed compounds unobvious. We are not faced with that question today.

Other cases, *e.g., In re Gyurik, 596 F.2d 1012, 1018, 201 USPQ 552, 557-58 (CCPA 1979)* ("no common-properties presumption rises from the mere occurrence of a claimed compound at an intermediate point in a conventional reaction yielding a specifically named prior art compound"), have qualified the original rule of the *Hass-Henze* cases, but it is clear that they have not enunciated a rule that, in order to make a *prima facie* case of obviousness, the examiner [**31] must show that the prior art suggests a new property discovered by applicant. In not accepting that principle today, as urged in the dissent, we are therefore not retreating from the recent trend of case law development or changing the law.

Another example of the lack of direct pertinence of a case quoted in the dissent is *May*, which the dissent cites as an example of the consistent line of decisions to the effect that "both structure and properties must be suggested in the prior art before a *prima facie* case of obviousness was deemed made." This case does not state that both structure and properties "must" be suggested. The claimed and prior art compositions were both disclosed as having analgesic activity; it was conceded that a *prima facie* case was made out, but the court concluded that applicants had rebutted the presumed expectation that structurally similar compounds have similar properties with a showing of an actual unexpected difference of properties between the claimed compound and the prior art. *574 F.2d at 1095, 197 USPQ at 611.* The applicant in that case thus made a showing that Dillon did not make in this case.

Properties must be considered [**32] in the overall evaluation of obviousness, and the lack of any disclosure of useful properties for a prior art compound may indicate a lack of motivation to make related compounds, thereby precluding a *prima facie* case, but it is not correct that similarity of structure and a suggestion of *the activity of an applicant's compounds* in the prior art are necessary before a *prima facie* case is established.

Conclusion

We affirm the Board's decision rejecting claims 2-14, 16-22, and 24-37.

AFFIRMED.

CONCUR BY: ARCHER (In Part)

CONCUR

ARCHER, Circuit Judge, with whom MARKEY and MICHEL, Circuit Judges, join, joining-in-part.

I join the majority opinion except to note that the comments on *In re Durden, 763 F.2d 1406, 226 USPQ 359 (Fed. Cir. 1985)*, are unnecessary to the decision of this case. The method claims were not rejected by the Board on the basis of that case and, in fact, were not separately considered by the Board. Until a rejection of a method claim based on *Durden* is appealed to this court and the issue fully briefed, it is premature to consider the scope and effect of [*699] *Durden*. Accordingly, I do not join this part of the majority [**33] opinion.

DISSENT BY: NEWMAN

DISSENT

NEWMAN, Circuit Judge, with whom COWEN, Senior Circuit Judge, and MAYER, Circuit Judge, join, dissenting.

The court today resolves certain disparities in the extensive body of precedent on the question of obviousness of new chemical compounds and compositions. How-

ever, these disparities are resolved in a way that is contrary to the weight and direction of this precedent, as embodied in over three decades of decision. The court departs from its considered development of the law governing patentability of new chemical compounds and compositions, and reinstates a rule of "structural obviousness". In so ruling the court not only rejects the weight of precedent, but also errs in the application of *35 U.S.C. §§ 101*, *102*, and *103*.

The majority's holding that *prima facie* obviousness of new chemical compounds and compositions is determined based only on structural similarity to prior art compounds and compositions having a known use[1] is reminiscent of the "Hass-Henze Doctrine" of earlier days. This doctrine was discarded thirty years ago, and although it resurfaced on occasion, its original sweep was superseded by many years of [**34] judicial analysis. Review of this analysis shows the courts' evolving understanding of the characteristics of chemical inventions, particularly the inseparability of chemical properties and chemical structure, and the legal consequences of this scientific fact.

1 The court allows one exception, as I shall discuss.

Judicial decisions over the past three decades established the general rule that the determination of *prima facie* obviousness of new chemical compounds and compositions and their uses can not be based on chemical structure alone, but must also include consideration of all their properties, including those discovered by the applicant. This rule had important procedural and substantive consequences during patent examination, for it determined the *kind* of evidence and proof that was required of a patent applicant. The ruling of this *in banc* court changes what must be proved in order to patent a new chemical compound or composition, and thus changes what is patentable.

The body of precedent establishing the burdens of modern patent examination was developed in judicial decisions applying the 1952 Patent Act. As I shall discuss in greater detail, when patent examination showed that the prior art suggested the inventor's [**35] new chemical structure and the inventor's newly discovered property and use, a *prima facie* case of obviousness of the new chemical compound or composition and its use was deemed made.

The inventor could, of course, argue that the differences from the prior art were such that a *prima facie* case was not made. And the inventor could rebut the *prima facie* case of obviousness, by showing some unexpected difference in structure or properties and use, not apparent from the teachings of the prior art. Such rebuttal was generally presented in the form of comparative experimental data, whereby the inventor demonstrated that the properties of his or her new chemical compound or composition achieved some unobvious or unexpected result or advantage, as compared with the actual properties of the prior art structure. This rebuttal often required the inventor to go beyond the general teachings in the prior art, and prove that the prior art compound did not, in fact, possess the specific property and advantage of the new compound or composition. Such proofs were invariably required when the prior art suggested the general property and use discovered by the applicant. Many dozens of decisions [**36] of the CCPA and this court illustrate this procedure, and the variety of factual circumstances in which it has been invoked.

In accordance with the court's *in banc* holding, [2] a new chemical compound or composition [*700] is not patentable even when the prior art does not suggest that the new chemical compound or composition would have the applicant's newly discovered property and use, unless the applicant makes the same showing that is required when the prior art does suggest the applicant's new property and use.

2 The majority holds that a *prima facie* case of obviousness is made whenever the structure of the applicant's new compound or composition (or mechanical device) is "obvious" from that shown in the prior art, independent of whether the prior art suggests or makes obvious the applicant's newly discovered property and use. The majority allows an exception for situations where the prior art gives no "reason or motivation to make the claimed invention", and duly makes clear that this means motivation to make the new compound or composition for the prior art use, not for the applicant's newly discovered use. This exception comes into play only when the prior art structure has no known utility; and a few such situations are reported, *e.g. In re Stemniski, 58 C.C.P.A. 1410, 444 F.2d 581, 170 USPQ 343 (CCPA 1971)* (prior art compounds used only as intermediates), and *In re Albrecht, 514 F.2d 1389, 185 USPQ 585 (CCPA 1975)* (no practical utility). Thus, according to the majority, when the prior art chemical compound or composition has no known use, the prior art provides no "reason or motivation" to make a structurally similar new compound or composition; and in such case the prior art would not make a *prima facie* case of obviousness based on structural similarity alone.

While I welcome any reduction in the sweep of the court's holding, this exception is of trivial impact. In most cases the prior art compound or

composition has some known use; and thus for most cases the majority's "motivation" test would be met based solely on similarity of structure. This is an important change of law. While the holdings of the prior law were not entirely consistent -- see the various CCPA opinions discussed *post* -- this *in banc* court now establishes the rule that will control all cases in the future.

[**37] The applicant is thus required to show "unexpected" properties and results, *whether or not* the prior art provides an expectation or suggestion of the properties and results disclosed in the patent application. And unless the applicant proves that the prior art structure does not actually possess the same unobvious property that the applicant discovered for the new structure, the court holds today that the new chemical compound or composition is not patentable. This is an incorrect application of the patent statute, and a rejection of the wisdom of precedent. Therefore, respectfully, I dissent.

I

THE LAW

A

The In Banc Issue

Diane M. Dillon appeals the decision of the PTO Board of Patent Appeals and Interferences, which had rejected all of Dillon's claims on the basis that "The claimed subject matter would have been *prima facie* obvious from the combined teachings of the references." The Commissioner, asking the court to choose between divergent decisions of the CCPA, stated the PTO position that *prima facie* obviousness should be based on chemical structure alone:

> If the prior art suggests an inventor's compound or composition per se, that compound [**38] or composition would be *prima facie* obvious, regardless of the properties disclosed in the inventor's application.

Commissioner's brief at 24.

The majority of the *in banc* court adopts the Commissioner's position, expressly rejecting the panel's position that the properties and use discovered by the applicant must be considered in connection with determination of the *prima facie* case under *section 103*, thereby overruling all prior decisions that so held. [3]

> 3 It is the role of the *in banc* court to resolve "a precedent-setting question of exceptional importance", *Fed. Cir. R. 35(a)*, and not simply to second-guess the panel on the facts of a particular case.

I would hold that determination of whether a *prima facie* case of obviousness has been made requires consideration of the similarities and differences as to structure *and* properties and utility, between the applicant's new compounds or compositions and those shown in the prior art. The *Graham [v. John Deere Co., 383 U.S. 1, 86 S. Ct. 684, 15 L. Ed. 2d 545, 148 USPQ 459 (1966)]* [**39] factors can not be ignored in determination of the *prima facie* case under *35* [*701] *U.S.C. § 103.* [4] As illustrated in precedent, pertinent considerations in determination of whether a *prima facie* case is made include the closeness of the prior art subject matter to the field of the invention, the motivation or suggestion in the prior art to combine the reference teachings, the problem that the inventor was trying to solve, the nature of the inventor's improvement as compared with the prior art, and a variety of other criteria as may arise in a particular case; all with respect to the invention as a whole, and decided from the viewpoint of a person of ordinary skill in the field of the invention.

> 4 *35 U.S.C. § 103.* A patent may not be obtained . . . if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. . . .

[**40] Of course not all these aspects will loom large in every case, but when present they must be considered. They are as pertinent to determination of the *prima facie* case as they are to the determination that is made after rebuttal evidence is adduced by the applicant. Structure alone, without consideration of the applicant's newly discovered properties, is an incomplete focus for consideration of these factors.

B

The Prima Facie Case

The *prima facie* case, as used in patent examination, means not only that the evidence of the prior art reasonably allows the examiner's conclusion of unpatentability, but also that the prior art compels such a conclusion as a matter of law, if the applicant produces no evidence to rebut it. *Black's Law Dictionary*, 1071 (5th Ed. 1979); *In re Piasecki, 745 F.2d 1468, 1472, 223 USPQ 785, 788 (Fed. Cir. 1984)*. See the discussion in *In re Mills, 47 C.C.P.A. 1185, 281 F.2d 218, 222 & n.2, 126 USPQ 513, 516 & n.2 (CCPA 1960)*, of the "presumption of unpatentability" based on prior art, a judicial usage before the term "*prima facie* case" appeared in CCPA and board decisions.

Patentability is determined, [**41] in the first instance, by examination of the patent application in the PTO. The prior art is searched by the patent examiner, who determines whether a *prima facie* case of unpatentability is made based on what was known in the prior art. *Piasecki, supra.* The *prima facie* rejection may be challenged by the applicant on grounds such as that the rejection is insufficiently supported, or incorrect, or based on illogical reasoning; or, if a *prima facie* case is made, evidence may be adduced in rebuttal. *In re Heldt, 58 C.C.P.A. 701, 433 F.2d 808, 811, 167 USPQ 676, 678 (CCPA 1970).* The *prima facie* case is not "set in concrete", and upon the submission of rebuttal evidence the determination of obviousness *vel non* is made on the entire record. *In re Rinehart, 531 F.2d 1048, 1052, 189 USPQ 143, 147 (CCPA 1976).*

The initial determination by the patent examiner is critical to further proceedings, for the presence or absence of a *prima facie* case of obviousness controls the need for the applicant to adduce rebuttal evidence of unobviousness. The procedure serves to clearly allocate the burdens of going forward and [**42] of persuasion as between the examiner and the applicant. *In re Johnson, 747 F.2d 1456, 1460, 223 USPQ 1260, 1263 (Fed. Cir. 1984).* It determines what the applicant must prove, and the kind of evidence the applicant must provide. Thus it determines what is patentable under the statute.

While many judicial decisions turn on the question of adequacy of the rebuttal evidence, the concern of the *in banc* court today is the issue of the *prima facie* case. For when, as here, no rebuttal evidence is presented, determination of the *prima facie* case is decision of the question of patentability.

As illustrated in Dillon's case, the question of whether there is a *prima facie* case of obviousness controls whether Dillon is required to prove that her newly discovered property of particulate (soot) reduction during combustion is not actually possessed by the prior art composition, when the prior [*702] art composition was not known or suggested to have this property. Dillon did not so prove, and the Commissioner urges that since Dillon's specification itself discloses that the prior art composition does possess this newly discovered property, the *prima facie* [**43] case based on structural similarity was not rebutted.

Heretofore, the courts generally recognized a controlling distinction between the two principal types of factual situations that arise when a patent applicant's new chemical compound or composition has a structure that is "similar" to chemical structures shown in the prior art: (1) those where the prior art suggests, at least in general terms, that the new chemical compound or composition will have the applicant's newly discovered property and use; and (2) those where it does not. These factual situations have had different consequences with respect to whether a *prima facie* case of obviousness was made. The difference turned on whether or not the structure *and* properties and use of a new chemical compound or composition were suggested in the prior art. The distinction determined whether the applicant was required to come forward with rebuttal evidence, which often was in the form of new technological information not known to the prior art, in order to establish an "unexpected" difference between the properties discovered by the applicant and those actually possessed by the prior art structure.

This distinction brought [**44] a consistent application of the law of *35 U.S.C. § 103* to the examination of chemical inventions, for it established the framework wherein the law was applied to the facts of each case. The court today rejects this distinction, holding *in banc* that it suffices to show *prima facie* obviousness whenever the prior art describes a similar chemical structure, provided only that the prior art gives some "reason or motivation" to make the claimed chemical structure, "regardless of the properties disclosed in the inventor's application", in the Commissioner's words. This position is contrary to the weight and direction of precedent, as I shall illustrate, and is contrary to the statutory imperatives of Title 35. [5]

5 The majority's insistence that it is merely reinstating the *status quo ante* can not be, of course, for the *in banc* holding that the applicant's newly discovered properties and use are not considered in determining *prima facie* obviousness under *section 103* (unless the prior art structure itself has no known use), such that the applicant must now prove that the prior art structure does not possess the newly discovered property and use, forecloses reliance on contrary precedent.

[**45] C

Precedent

It is critical to an effective patent system that there be consistent law, consistently applied, despite the great diversity of technologies that the patent system must serve. The law that is applied in determining whether a *prima facie* case of obviousness is made, as to new chemical compounds and compositions and their uses, has evolved from analysis of many different factual situations. The large number of decisions on this issue, of this court and the CCPA, produced a rich body of precedent.

The following outline of precedent is chronological, and has its roots in cases decided before enactment of the 1952 Patent Act.

For a while, during an earlier period of modern chemistry, "structural obviousness" alone was deemed to create a presumption of unpatentability. Leading decisions were *In re Hass, 31 C.C.P.A. 895, 141 F.2d 122, 127, 130, 60 USPQ 544, 548, 552 (CCPA 1944)* and *In re Henze, 37 C.C.P.A. 1009, 181 F.2d 196, 85 USPQ 261 (CCPA 1950)*. In these decisions the CCPA held that there was a presumption of obviousness based solely on the close similarity of structure of chemical homologs, [6] regardless of the new property [**46] and use that the applicant discovered. Thus a new compound, if a homolog of a known compound, was not patentable unless the applicant [*703] overcame this presumption by showing that there was in fact a significant and unexpected difference in the actual properties [7] of the new compound as compared with the actual, albeit unknown to the prior art, properties of the known homolog:

> The burden is on the applicant to rebut that presumption by a showing that the claimed compound *possesses* unobvious or unexpected beneficial properties not actually *possessed* by the prior art homologue.

Henze, 181 F.2d at 201, 85 USPQ at 265 (emphases in original). This difference in properties was required to be shown to exist, whether or not the prior art suggested the property and utility discovered by the inventor for the new compound, and whether or not there was any reason to expect the prior art compound to have that property or utility. The court held:

> It is immaterial that the prior art homologue may not be recognized or *known* to be useful for the same purposes or to possess the same properties as the claimed compound.

[**47] *Id.* (emphasis in original).

6 A homolog is a chemical compound that differs from another compound only by one or more methylene groups. An "adjacent" homolog differs by precisely one methylene group. *Henze, 181 F.2d at 200, 85 USPQ at 264.*

7 As used in these decisions "property" and "utility" refer to the utilitarian property that complies with *35 U.S.C. § 101*, and not to physical or chemical parameters not pertinent to patentability. *In re Krazinski, 52 C.C.P.A. 1447, 347 F.2d 656, 659, 146 USPQ 25, 28 (CCPA 1965)* ("a 'utility' is really a manifestation of a 'property'").

The courts soon expressed dissatisfaction with this rule. See the discussion, and cases cited, in *In re Mills, 47 C.C.P.A. 1185, 281 F.2d 218, 126 USPQ 513 (CCPA 1960)*. In *Mills* the CCPA limited the *Henze* presumption to adjacent homologs, and rejected the PTO's interpretation of *Henze* as establishing a "legal presumption" of obviousness. [**48] The court held that homology alone does not create an inference of unpatentability or shift the burden of persuasion, but is simply a fact "which must be considered with all other relevant facts before arriving at the conclusion of 'obviousness' specified in *35 U.S.C. § 103*." *Id. 281 F.2d at 224, 126 USPQ at 518.*

Throughout the 1960's, and thereafter, the CCPA stressed that properties as well as structure were material to the patentability of new chemicals, and *must* be considered. The case of *In re Papesch, 50 C.C.P.A. 1084, 315 F.2d 381, 137 USPQ 43 (CCPA 1963)* is often cited for its statement that "a chemical compound and all of its properties are inseparable". *Id. at 391, 137 USPQ at 51.* See *Papesch* for citation of a number of decisions of the CCPA and other courts after *Henze*, illustrating judicial understanding that chemical structure is not severable from chemical (including biochemical) properties in patentability determinations; for it is the properties that determine the utility that is requisite to patentability.

The *Papesch* court rejected the principle that this court now adopts, for in [**49] *Papesch* the court held that the prior art must disclose "at least to a degree" the applicant's desired property and utility. The court said:

> The other factor of importance . . . is that the prior art disclosure was not merely of a structurally similar compound but also, at least to a degree, of *the same desired property* relied on for the patentability of the new compound. Such an "other factor" must of course be considered because it bears on the obviousness of the compound, which is, realistically and legally, a composite of both structure and properties.

Id. at 392, 137 USPQ at 52 (emphasis in original). The majority today disposes of this passage, long the bulwark of chemical practice, as "a brief mention", and "irrelevant" to the *prima facie* case. This passage has been reinforced myriad times after its appearance in *Papesch*, and has been a foundation of the *prima facie* case in examination of chemical patent applications for obviousness in terms of *section 103*.

Although the CCPA and the Patent Office at the time of *Papesch* did not generally use the terminology "*prima facie* case", the CCPA's holding that a compound is "realistically [**50] and legally, a composite of both structure and properties", underlies the requirement that both structure and properties be considered. *Papesch* and its [*704] extensive progeny establish that the prior art disclosure should be "at least to a degree, of the same desired property", *id.*, even for a homolog. [8] Absent such disclosure, the *presumption* of unpatentability did not arise. [9] This requirement, that both structure and properties be considered, simply continued when the terminology of the *primá facie* case was adopted to describe a legally sufficient case of obviousness under *section 103*.

8 Dillon's tetra-orthoester is not a homolog of the tri-orthoester. Nor is it an isomer. The majority opinion simply describes the tri-orthoester and tetra-orthoester structures as having "a sufficiently close relationship", without the precision of past decisions.

9 In Dillon's case such disclosure was absent. It is undisputed that Dillon's property of soot-reduction during combustion was not disclosed for the prior art compositions.

[**51] In *In re De Lajarte, 52 C.C.P.A. 826, 337 F.2d 870, 143 USPQ 256 (CCPA 1964)* the same principle was applied to a new composition. The CCPA held that similarity of chemical composition alone did not place on the applicant the obligation of proving that the prior art composition did not possess the applicant's newly discovered property and utility:

> In the total absence of evidence in the record to indicate that the amber glass disclosed by Lyle would be expected to have desirable electrical insulating properties, we can find no justification for placing the burden on applicant to conduct experiments to determine the insulating properties of the colored glass disclosed by Lyle.

Id. at 874, 143 USPQ at 259. As in the modern usage of the *prima facie* case, the court held that the prior art must suggest both similar structure and property before the burden shifted to the applicant to prove unexpected differences. De Lajarte's claims were to a novel glass composition having insulating properties. The prior art (Lyle) reference showed a glass composition having what the examiner called "very slight differences" in structure, and the [**52] properties of amber color and durability. The court said:

> If one were making a colorless glass free of carbon and sulfur, there would be little reason for using the Lyle formula since it was primarily designed to enhance color stability. In the absence of any showing why it would be obvious to modify Lyle's glass, a "103 rejection" must be reversed.

Id. at 875, 143 USPQ at 259.

In *In re Ruschig, 52 C.C.P.A. 1238, 343 F.2d 965, 145 USPQ 274 (CCPA 1965)* the court again observed that the invention "indeed, is a 'conjunction of utility and product', though claimed as new compounds found *to have* the desired biological effect". (Emphasis in original.) The court criticized the PTO position that "the examiner and the board thought the compounds, looked upon as mere chemical formulae, would have been obvious". *Id. at 973, 145 USPQ at 281.* The court stated:

> On the obviousness issue, the vague "basket" disclosure of possible uses in the French and Swedish patents and the equally vague disclosure of the Martin patent are unimportant. What is important is the fact that the utility *discovered by the appellants* is not disclosed [**53] in the prior art.

Id. at 977, 145 USPQ at 285 (emphasis in original). [10]

10 In *Ruschig* the court discussed the Commissioner's position that claims to compounds that are structurally similar to known compounds should "define" the newly discovered property on which patentability was based. The CCPA rejected such limitation, stating that "valuable inventions should be given protection of value in the real world of business and the courts". *343 F.2d at 979, 145 USPQ at 286.*

The majority of the court today, remarking that "the composition claims are not limited to [Dillon's] new use", hints that the result might be different if they were. *Ruschig* and other cases consistently rejected the proposition that claims to new compounds and compositions *must* contain a limitation to a specific use. Any change in this long-established practice requires careful

thought. The majority also holds that Dillon's claim recitation of "a sufficient amount of [orthoester] to reduce the particulate emissions" is ineffective as a use limitation, adding uncertainty to claim draftsmanship.

[**54] That the utility discovered by the applicant must be at least suggested in the prior art, in order to establish *prima facie* unpatentability [*705] of new compounds and compositions that are structurally similar to known chemicals, is the common thread that ties most of the decisions of the CCPA and the Federal Circuit. I do not attempt to include all of the cases that illustrate this point. I do, however, include representative cases of divergent holding, and those that appear to be relied on by the majority.

For example, the Commissioner cites *In re de Montmollin, 52 C.C.P.A. 1287, 344 F.2d 976, 145 USPQ 416 (CCPA 1965)* as supporting authority. In *de Montmollin* the claimed invention was a new compound, described by the applicant as useful for dyeing wool and cotton. The reference showed structurally similar compounds useful for dyeing wool. The court concluded that the additional ability of de Montmollin's claimed compound to dye cotton was not "sufficient to render the subject matter as a whole unobvious". *Id. at 979, 145 USPQ at 417-18.* This case simply illustrates determination of obviousness by comparing the structures and properties taught [**55] in the prior art with those disclosed by the applicant, and bringing judgment to bear on "the subject matter as a whole." *Id.* It does not support the position that a *prima facie* case under *section 103* is made on structural similarity, regardless of the applicant's newly discovered property and use.

The principle that both structure and properties must be considered in connection with the *prima facie* case was directly treated in *In re Rosselet, 52 C.C.P.A. 1533, 347 F.2d 847, 146 USPQ 183 (CCPA 1965)*, where the court held that "gross similarity" of chemical structure *and* the same area of pharmacological activity constituted a *prima facie* case of obviousness:

> We agree with appellant's admonitions against deciding questions of chemical obviousness on the basis of structure *alone*, whether by means of a "mechanistic overlay" or otherwise. However, we think appellants have failed to present adequate evidence to overcome a *prima facie* showing of obviousness by reason of the admitted "gross structural similarities" of the art compounds, coupled with the fact those compounds are shown to have utility *in the same area of pharmacological* [**56] *activity*.

Id. at 850, 146 USPQ at 185 (emphases in original).

Other decisions during this period illustrate varying judicial emphases, depending on the facts. When the compounds were quite closely related in chemical structure, as in the homologs and analogs of *In re Riden, 50 C.C.P.A. 1411, 318 F.2d 761, 138 USPQ 112 (CCPA 1963)*, or the position isomers of *In re Mehta, 52 C.C.P.A. 1615, 347 F.2d 859, 146 USPQ 284 (CCPA 1965)*, the court gave greater evidentiary weight to the structural similarities than when the structures were less directly comparable. However, the court regularly admonished that:

> A compound is not, however, merely a structural formula; its properties as part of the whole must be considered.

Id. at 864, 146 USPQ at 287.

In *In re Lunsford, 53 C.C.P.A. 986, 357 F.2d 380, 148 USPQ 716 (CCPA 1966)* the court listed eight recent decisions in which it had rejected the Patent Office's position of "comparison of structures only":

> Just how one finds the compounds "obvious" in the first instance, the examiner does not say, but apparently he envisions a comparison of structures [**57] *only*. That such an approach is not sanctioned by this court, although concededly the law was less well defined in June 1961, the date of the Examiner's Answer, can be seen, e.g., in *In re Krazinski, [52 C.C.P.A. 1447], 347 F.2d 656, 146 USPQ 25* [(CCPA 1965)]; *In re Ruschig, [52 C.C.P.A. 1238], 343 F.2d 965, 145 USPQ 274* [(CCPA 1965)]; *In re Ward, [51 C.C.P.A. 1132], 329 F.2d 1021, 141 USPQ 227* [(CCPA 1964)]; *In re Lunsford, [51 C.C.P.A. 1000], 327 F.2d 526, 140 USPQ 425* [(CCPA 1964)]; *In re Riden, Jr., [50 C.C.P.A. 1411], 318 F.2d 761, 138 USPQ 112* [(CCPA 1963)]; *In re Papesch, [50 C.C.P.A. 1084], 315 F.2d 381, 137 USPQ 43* [(CCPA 1963)]; *In re Petering, [49 C.C.P.A. 993], 301 F.2d 676, 133 USPQ 275* [(CCPA 1962)]; *In re Lambooy, [49 C.C.P.A. 985], 300 F.2d*

> *950,* [*706] *133 USPQ 270* [(CCPA 1962)].

Id. at 382 n.2, 148 USPQ at 718 n.2 (emphasis in original). The court reiterated:

> The discovered activities or properties are *part of* the things claimed, the compounds. . . . We, like [**58] the Patent Office, are required by the clear wording of *section 103* to regard it as a relevant portion of the invention "as a whole."

Id. at 384, 148 USPQ at 720 (emphasis in original).

In a companion case, *In re Lunsford, 53 C.C.P.A. 1011, 357 F.2d 385, 148 USPQ 721 (CCPA 1966)*, the court again stressed that determination of obviousness must be based on both structure and properties. The court quoted the Patent Office position, in the examiner's words:

> The argument that the "subject matter as a whole" under *35 U.S.C. 103* includes the compound and its utility is considered to be without merit.

Id. at 391, 148 USPQ at 725. The court once more firmly rejected that position:

> It is reasonably clear that the examiner considered only the difference in *structures* between the claimed compounds and the prior art compounds.
>
> Appellant was entitled to have differences between the claimed invention, *the subject matter as a whole*, and the prior art references of record evaluated.

Id. (emphases in original). Referring to *35 U.S.C. § 103*, the court wrote: [**59]

> It is not believed that [a person of ordinary skill in the art] would consider only the *structures* of the compounds[.]

Id. (emphasis in original).

These judicial analyses, written before the terminology of the *prima facie* case became routine in patent examination, nonetheless placed the burden on the examiner to come forward with prior art sufficient to support any purported suggestion of the applicant's properties:

> Moreover, as a matter of law under *35 U.S.C. 103*, the examiner must substantiate his "suspicions" on the basis of facts drawn from proper prior art. The issue to be resolved requires more than "suspicions;" it requires *facts*.

Id. (footnote omitted, emphasis in original).

The CCPA put "structural obviousness" in perspective when, again receiving this argument from the Commissioner, the court commented on the large numbers of yet-unmade compounds that would be "structurally obvious" to a chemist:

> The solicitor has reargued in his brief the question of the obviousness of chlorpropamide to a chemist as a compound, that is, what we sometimes refer to as its "structural obviousness. [**60] " This is not a matter of dispute. We think appellant would concede that all 1,237,464 compounds he claims to be within the Ruschig et al. disclosure or the 38,556 compounds he claims to be within the broadest patent claim, are structurally obvious.

In re McLamore, 54 C.C.P.A. 1544, 379 F.2d 985, 989, 154 USPQ 114, 117 (CCPA 1967). Indeed, few of today's new chemicals have such imaginative structure that structurally similar compounds are not to be found in the prior art. [11] Nonetheless, the reasoning rejected in *McLamore* appeared two years later in the CCPA decision *In re Mod*.

11 In 1980 the Supreme Court noted that over 4,848,000 compounds had been listed by the Chemical Abstracts Service. *Dawson Chemical Co. v. Rohm & Haas Co., 448 U.S. 176, 221 n.23, 100 S. Ct. 2601, 65 L. Ed. 2d 696, 206 USPQ 385, 407 n.23 (1980)*. In 1990 the ten millionth specific compound had been entered in the register. *Chemical & Engineering News*, February 26, 1990, p. 30. This count is of characterized compounds, and does not include theoretical permutations and combinations such as those calculated in *McLamore*.

[**61] *In re Mod, 56 C.C.P.A. 1041, 408 F.2d 1055, 161 USPQ 281 (CCPA 1969)* was described by the Commissioner as "on all fours" with Dillon's facts. Mod's new compounds were adjacent homologs and isomers of known compounds that were described in the prior art as insecticides. Mod's compounds had "antimicrobial activity against a variety of micro-organisms such as bacteria, yeast, and molds". *Id. at 1055, 161 USPQ at 282*. The court held Mod's compounds unpatentable [*707] for obviousness, referring to the close similarities of structure and remarking that Mod "did not deny" that his new compounds had insecticidal activity. The court in *Mod* did not discuss a "*prima facie* case", but stated that it was not sufficient for patentability that the antimicrobial activity of Mod's new compounds was unknown to the prior art. *Id. at 1057, 161 USPQ at 283*. In a decision analogous to the majority's holding today, the CCPA held that because Mod's new compounds would have been obvious for the prior art use as insecticides, they were unpatentable despite Mod's discovery of a "significant" new property. *Id.*

Mod has often been [**62] distinguished. See, e.g., *In re Albrecht, 514 F.2d 1389, 185 USPQ 585 (CCPA 1975)*, wherein the court directly contradicted *Mod*, as follows:

> We are of the opinion that a novel chemical compound can be *nonobvious* to one having ordinary skill in the art notwithstanding that it may possess a known property in common with a known structurally similar compound.

Id. at 1395-96, 185 USPQ at 590 (emphasis in original). With rare exceptions *Mod* has not been followed; until today. [12]

12 The facts in *Mod* are indeed on all fours with the facts now before us. In *Mod* the court held that the apparently shared property of insecticidal activity sufficed to make Mod's new (but structurally similar) compounds unpatentable for obviousness, despite Mod's discovery that his new compounds had the new property of antimicrobial activity. In Dillon's case the court holds that the presumptively shared property of water sequestration suffices to make Dillon's new (but structurally similar) compositions unpatentable for obviousness, despite Dillon's discovery that her new compositions have the new property of soot reduction. In neither case did the prior art suggest or make obvious the applicant's newly discovered property. The absence of the term "*prima facie* case" from the *Mod* opinion does not distinguish it from the majority's holding today. A conclusion of obviousness must be legally sufficient under the statute, and for both Mod and Dillon the courts hold that on similar facts this conclusion is compelled as a matter of law.

[**63] Continuing the chronological review: In *In re Jones, 56 C.C.P.A. 1293, 412 F.2d 241, 162 USPQ 224 (CCPA 1969)* the court upheld the rejection of composition claims to a blend of polypropylene and an asbestos filler as *prima facie* obvious, for the reason that "the prior art suggests the combination generally for the primary purpose (*i.e.*, improved structural rigidity) for which it was made." *Id. at 244, 162 USPQ at 226*. Comparative data were properly required, for both structure and properties were suggested in the prior art.

The procedural and substantive rigor of the *prima facie* case in patent examination is again illustrated in *In re Godron, 57 C.C.P.A. 1289, 428 F.2d 854, 166 USPQ 327 (CCPA 1970)*, wherein the court held that since a *prima facie* case of obviousness was not made as to a new glass composition having new and unobvious properties, it was unnecessary for the applicant to prove whether the prior art composition had the same property (the ability to wet graphite) as the applicant's composition:

> We agree that it is improper to require comparative evidence where a reference is devoid of any suggestion of [**64] the claimed invention.

Id. at 855, 166 USPQ at 329.

This procedure was similarly applied to chemical process claims. For example, in *In re Freed, 57 C.C.P.A. 1089, 425 F.2d 785, 165 USPQ 570 (CCPA 1970)* the claimed invention was a single-step process for producing calcium pantothenate. The CCPA held that the examiner had failed to support a *prima facie* case, since the prior art disclosed only a two-step process. Since a *prima facie* case was not made, the court held that it was unnecessary to show unexpected results. *Id. at 788* & n.5, *165 USPQ at 572* & n.5. *See also, e.g., In re Alul, 468 F.2d 939, 943, 175 USPQ 700, 703-04 (CCPA 1972)* (since *prima facie* case not established, no need to consider contentions of unobvious results).

In *In re Kuderna, 57 C.C.P.A. 1078, 426 F.2d 385, 165 USPQ 575 (CCPA 1970)* the board held that a claimed carbamate compound, having utility as an insec-

ticide, was *prima facie* obvious in view of structurally similar known carbamates described in the prior art as useful as insecticides. The court held that the applicant's showing [*708] of superior results demonstrated [**65] unobviousness, in view of the entirety of the relevant teachings of the prior art and the absence of anything "on which to base a prediction of the comparative insecticidal activities" of these carbamate compounds. *Id. at 390, 165 USPQ at 579.*

In *In re Hoch, 57 C.C.P.A. 1292, 428 F.2d 1341, 166 USPQ 406 (CCPA 1970)* the reference described a known compound and its use for "treatment of plant diseases"; the court held that a *prima facie* case of obviousness was made as to the applicant's structurally similar compound disclosed to be useful as a herbicide, stating:

> On the face of it, "treatment of plant diseases" could mean usefulness in controlling plant-infesting organisms. . . . We are thus not persuaded that herbicidal properties are "contraindicated" by the [prior art] patent.

Id. at 1343, 166 USPQ at 408-09. Hoch simply illustrates the court's consideration of the closeness of the chemical structures (the only difference was between an acid and its ethyl ester) *and* the court's stated uncertainty about the relationship of the properties shown in the prior art and those disclosed by the applicant, in deciding [**66] whether a *prima facie* case was made.

The classical reasoning of the *prima facie* case is again illustrated for composition claims in *In re Susi, 58 C.C.P.A. 1074, 440 F.2d 442, 169 USPQ 423 (CCPA 1971).* A composition comprising a polymer and a stabilizer was held *prima facie* obvious because a similar combination was suggested in the prior art for the same purpose, and Susi's stabilizer compounds were similar in structure to known compounds having the known property of stabilizing similar polymers. *Id. at 444, 169 USPQ at 425.* Thus a showing of unobvious results was properly required, for both structure and properties of the new composition were suggested in the prior art. [13]

> 13 There is extensive precedent relating to chemical compositions as well as chemical compounds; see the sampling herein. The statement by the majority that its *in banc* holding does not "necessarily" apply to "all cases" of chemical compositions will not add clarity and stability to the law. The court's holding today must be followed by patent examiners and patent applicants, and while I dissent from it, it is essential that inventors and examiners, producing and processing 150,000 new patent applications annually, know the law and practice that will control patentability.

[**67] Another routine illustration of the *prima facie* case based on similarity of both structure and properties, between the prior art and the claimed compound, is seen in *In re Ackermann, 58 C.C.P.A. 1405, 444 F.2d 1172, 170 USPQ 340 (CCPA 1971).* The board had rejected claims to an optical brightening agent as *prima facie* obvious from references describing structurally similar compounds having pronounced fluorescence and optical brightening properties. The CCPA agreed that a *prima facie* case had been made, since the "references also suggest that the art would expect the class of compounds as a whole to possess the general property of optical fluorescence"; but the court reversed on the question of sufficiency of the rebuttal evidence. *Id. at 1176, 170 USPQ at 342-43.*

In *In re Stemniski, 58 C.C.P.A. 1410, 444 F.2d 581, 170 USPQ 343 (CCPA 1971)* the court again held that similarity of structure alone was insufficient for *prima facie* unpatentability. The claimed new compounds were analogs of known diaryl-tin compounds, and the known compounds were described as having no established utility, although utilities were suggested by the [**68] court. The court observed that the prior art provided no motivation to make the claimed compounds for applicant's purpose, although "one of ordinary skill would suppose the properties or potential uses of the two groups of compounds would be similar." *Id. at 585, 170 USPQ at 347.* [14]

> 14 As pointed out at n.2, *supra*, the facts illustrated in *Stemniski*, where the prior art compounds assertedly had no known use, are the only circumstance wherein the majority allows that structural similarities alone do not make a *prima facie* case of obviousness under *section 103.* While a chemical compound may be of solely theoretical interest, it is hard to imagine a composition (or a mechanical device) with no known use; thus this exception has scant practical significance with respect to compositions and mechanical devices.

[*709] The *Stemniski* court remarked on "this court's failure to render consistent precedent" on the issue of structural obviousness, *id. at 585, 170 USPQ at 347*, stating that [**69]

> *Henze*, its predecessors and its progeny have met with their share of criticism over

> the years, both in this court, in other courts, and elsewhere.

Id. at 587, 170 USPQ at 348 (footnote omitted). The court confronted the Commissioner's argument on the sufficiency of structural similarity alone, and held:

> To the extent that *Henze* and *Riden* are inconsistent with the views expressed herein, they no longer will be followed, and are overruled.

Id. at 587, 170 USPQ at 348.

In *In re Murch, 59 C.C.P.A. 1277, 464 F.2d 1051, 175 USPQ 89 (CCPA 1972)* the claims were directed to new polymer compositions described as having certain properties of toughness. A *prima facie* case was deemed made based on similar properties of toughness described for similar polymer compositions in the prior art; and was held rebutted based on evidence of improved weld line toughness. *Id. at 1056, 175 USPQ at 92.* Although the majority today cites *Murch* as supporting authority for "structural obviousness", the court in *Murch* distinguished *Mod* and *de Montmollin*, and reaffirmed reliance on *Papesch*. *Murch* is remote from [**70] a holding of *prima facie* obviousness based on structural similarity alone.

In *In re Freeman, 474 F.2d 1318, 177 USPQ 139 (CCPA 1973)* the court again distinguished *Mod* and *de Montmollin*, which had again been cited by the Commissioner for the position that structural similarity alone suffices to support a *prima facie* case of obviousness. The court stated:

> We have recently pointed out that the rule derived from *Mod* and *de Montmollin* by the Patent Office is not supported by those cases.

Id. at 1322, 177 USPQ at 142. The court discussed the principle of the *prima facie* case as applied to *section 103*, explaining that if the evidence of obviousness of the claimed subject matter

> is strong enough to establish a *prima facie* case, i.e., one which would prevail in the absence of rebuttal evidence, we must go on to examine the evidence of non-obviousness before the legal conclusion called for by *section 103* can be reached.

Id. The court stressed the need to consider all the evidence.

The claimed invention in *In re Mochel, 470 F.2d 638, 176 USPQ 194 (CCPA 1972)* was [**71] sodium glass compositions containing magnesium oxide, a component known for use in lithium glass compositions. The court held:

> No suggestion appears in the record of a purpose for including MgO in sodium glasses and no teaching of identical effects of secondary components in sodium and lithium glasses. We do not consider it "fair to assume" [quoting the examiner] such identity. We cannot, therefore, sustain the finding of *prima facie* obviousness.

Id. at 641, 176 USPQ at 196.

In *In re Cescon, 474 F.2d 1331, 177 USPQ 264 (CCPA 1973)* the court reversed the rejection of compound claims, where the board had relied on known methods of making the compounds as evidence of obviousness, and the examiner had "totally disregarded" the showings of improved properties. The court stated:

> It has long been our position that a compound and its properties are inseparable and that no property can be ignored in determining patentability over the prior art.

Id. at 1334, 177 USPQ at 266. The court found that the prior art did not suggest a relationship between the change in chemical structure and the applicant's [**72] phototropic properties. The court stated that it was insufficient that this type of chemical modification was known:

> Nothing in the prior art suggests any relationship between ortho substitution in the 2-phenyl ring [the structural difference from the prior art] and the properties [*710] disclosed and illustrated in appellant's specification.

Id. at 1334, 177 USPQ at 266-67. The court quoted with approval the following statement from *In re Larsen, 49 C.C.P.A. 711, 292 F.2d 531, 533, 130 USPQ 209, 210 (CCPA 1961), cert. denied, 370 U.S. 936, 8 L. Ed. 2d 806, 82 S. Ct. 1580 (1962)*:

> Since there was nothing to indicate that the compounds, when made, would have these properties, it was not obvious to make the compounds. In such a case the allowance of claims to the compounds must depend on the proposition that it was unobvious to conceive the idea of producing them, within the meaning of Title *35 U.S.C., Section 103.*

Cescon, 474 F.2d at 1334, 177 USPQ at 266.

These cases illustrate the CCPA's continuing rejection of the "structural obviousness" [**73] theme, on the rationale that unless there was a reason or motivation shown in the prior art to make the particular structural change that the applicant made, in order to achieve the properties that the applicant was seeking, "it was not obvious to make the compounds." *Id.* As the court explained, the "idea" of new compounds is not separable from the properties that were sought by the inventor when making the compounds. Structure and properties are essential components of "the invention as a whole", in the words of *section 103*.

In *In re Taborsky, 502 F.2d 775, 183 USPQ 50 (CCPA 1974)* the prior art described a large number of isomers and analogs of the applicant's compounds. Taborsky's products were "halogen" compounds, discovered to be useful as selective piscicides against brown bullhead fish and as larvicides to control sea lampreys. The prior art compounds were described as controlling gastropods and snails. The *Taborsky* decision illustrates the care with which the court compared, compound by compound, the applicant's and the prior art's disclosure of structures *and* properties. As to the applicant's chlorine, bromine, and iodine analogs, the court remarked [**74] that it was unnecessary to pass on the issue of whether a *prima facie* case were made by the prior art's disclosure of related compounds containing these three halogens, because such case, if made, had been overcome by evidence of the comparative effectiveness of these compounds as larvicides to control sea lampreys. *Id. at 781, 183 USPQ at 55.* As to the fluorine analogs, the *Taborsky* court held that the board's holding of *prima facie* obviousness "because of structural similarity to the cited prior art" could not stand, since there was "no motivation to make the proposed molecular modifications needed to arrive at appellant's claimed fluoro-substituted compounds." *Id. at 780-81, 183 USPQ at 55.* The court held that it was not necessary for the applicant to compare actual properties of the new and the prior art compounds:

> Since we hold that the prior art of record fails to establish that the fluoro-substituted compounds recited in appellant's claims are *prima facie* obvious, it is unnecessary to consider any comparative evidence with respect to the properties of these compounds.

Id. at 781, 183 USPQ at 55. [**75]

Only when the *prima facie* case was established by the prior art was the applicant required to develop comparative evidence. It is as much an obligation to avoid imposing unnecessary burdens on the applicant, and on the Patent Office, as it is to assure compliance with the law. *See, e.g., In re Seigneurin, 474 F.2d 1020, 1023, 177 USPQ 257, 259 (CCPA 1973)* (since no *prima facie* case of obviousness was made for a catalyst component of a chemical process, "that concludes the matter"); *In re Fischer, 484 F.2d 961, 963-64, 179 USPQ 304, 306 (CCPA 1973)* (no *prima facie* case, therefore no need to show unexpected results of claimed method). While the CCPA did not always decline to consider such evidence when it was already of record, the principle underlying orderly patent examination is that the burden in the first instance is on the examiner to establish that the claimed invention is *prima facie* unpatentable based on the prior art.

The no-utility exception that is recognized in the majority opinion is seen in the [*711] following case. In *In re Albrecht, 514 F.2d 1389, 185 USPQ 585 (CCPA 1975)* (*Albrecht* I) [**76] the prior art compounds were described as anesthetics that had the disadvantage of being skin irritants and therefore were not of practical use. Albrecht's new compounds were antiviral agents. The claims were rejected as *prima facie* obvious based on similarity of structure; in the examiner's words:

> Where such closely structurally related compounds are concerned, actual unexpected *differences* in properties are required to overcome a prima facie case of obviousness. Even though the present compounds may exhibit an unexpected

> property as anti-virals, they are obvious as anesthetics.

Id. at 1392, 185 USPQ at 587 (emphasis in original). The Commissioner argued that Albrecht must prove that his new compounds were not anesthetics. The court disagreed, holding that the properties shown in the prior art did not provide the "necessary impetus" to make Albrecht's compounds, *id. at 1396, 185 USPQ at 590*. The court also reaffirmed that properties must always be considered:

> This court has several times recently expressed its position on the role of the properties of chemical compounds in assessing their obviousness under *§ 103*. [**77] See *In re Taborsky, 502 F.2d 775, 183 USPQ 50 (CCPA 1974)*; *In re Blondel, 499 F.2d 1311, 182 USPQ 294 (CCPA 1974)*; see further *In re Murch, [59 C.C.P.A. 1277], 464 F.2d 1051, 175 USPQ 89 (CCPA 1972)*; *In re Lintner, [59 C.C.P.A. 1004], 458 F.2d 1013, 173 USPQ 560 (CCPA 1972)*.

Id. at 1394, 185 USPQ at 588-89. The court observed that Albrecht's newly discovered antiviral property is "totally dissimilar" to the properties disclosed for the prior art compounds, and is "not to be ignored":

> A newly discovered activity of a claimed novel compound which bears no material relationship to the activity disclosed for the prior art analogs is further evidence, not to be ignored, of the nonobviousness of the claimed invention.

Id. at 1396, 185 USPQ at 590. The court reversed the rejection.

In *In re Albrecht, 514 F.2d 1385, 185 USPQ 590 (CCPA 1975)*, decided the same day, the court held, as to other related compounds, that a *prima facie* case was made based on structural similarity to the compounds of the same prior art reference as in *Albrecht* I. The court disposed [**78] of the Commissioner's position that the applicant should have provided comparative data with the anesthetic property of the prior art compounds, as follows:

> There seems to be little doubt that the Patent and Trademark Office would not have entertained such a comparison if initiated by appellants because they have no support in their specification for use of the claimed compounds as anesthetics. See *In re Davies, 475 F.2d 667, 177 USPQ 381 (CCPA 1973)*.

Id. at 1389, 185 USPQ at 593. The court correctly observed that comparative data, when required to rebut a *prima facie* case, should relate to the new property and use discovered by the applicant, not an unrelated known use of the prior art products.

In *In re Lamberti, 545 F.2d 747, 192 USPQ 278 (CCPA 1976)* certain new compounds having germicidal activity were rejected as *prima facie* obvious over structurally similar compounds described as biological toxicants, a use that the court found may include germicidal activity, although not necessarily. *Id. at 750, 192 USPQ at 280*. Rebuttal evidence was adduced and found insufficient. The court concluded: [**79]

> In view of the foregoing, and considering the claimed invention as a whole vis-a-vis the evidence produced by the PTO, which shows both a close structural similarity between the prior art compounds and the claimed compounds and a close similarity between the *disclosed* uses, we hold that appellants' invention would have been obvious to one of ordinary skill in the pertinent art.

Id. at 751, 192 USPQ at 281 (emphasis added).

The majority has cited *In re Shetty, 566 F.2d 81, 195 USPQ 753 (CCPA 1977)*. Shetty's [*712] new compounds were homologs of known compounds that were described in the prior art as antiviral agents, whereas Shetty's compounds were discovered to have appetite-suppressant activity. Diverging from the weight of its precedent, the court held that a *prima facie* case was made based on the close similarities of chemical structure. Since Shetty did not prove that there were actual differences in properties, the composition claims were not allowed.

However, the *Shetty* court allowed the process claims (to the new use), [15] without requiring such proof. The court said, following precedent:

> The [**80] Patent Office has failed to show a reasonable expectation, or some predictability, that [a reference] compound would be an effective appetite suppressant if administered in the dosage disclosed by [another reference].

Id. at 86, 195 USPQ at 756. In contrast, the majority today affirms the rejection of Dillon's claims, process and composition, although such "expectation" or "predictability" was, without dispute, absent.

> 15 A new use is claimed as a process, in accordance with *35 U.S.C. § 100(b)*:
>
> > The term "process" means process, art or method, and includes a new use of a known process, machine, manufacture, composition of matter, or material.

Despite the occasional aberration illustrated by *Shetty*, the CCPA decisions became remarkably consistent in requiring that both structure and properties must be suggested in the prior art before a *prima facie* case of obviousness was deemed made, whether for composition claims or process [**81] claims. *E.g., In re May, 574 F.2d 1082, 197 USPQ 601 (CCPA 1978)*, wherein the prior art compounds were isomers of May's compounds, and showed the same analgesic activity. A *prima facie* case was conceded as to the composition and the process claims, and overcome by rebuttal evidence of the non-addictiveness of May's compounds, an attribute that was shown to be unpredictable.

Similar reasoning was applied to claims to a new use of a known compound in, *e.g., In re Herschler, 591 F.2d 693, 200 USPQ 711 (CCPA 1979)*, wherein the use of the known compound DMSO (dimethyl sulfoxide) to enhance steroid penetration through skin tissue was held by the board to be *prima facie* obvious from references showing DMSO in hair lotion preparations that may contain estrogenic hormones (steroids). The court reversed, holding that "the references do not provide any impetus to do what appellant has done". *Id. at 702, 200 USPQ at 718-19.* In the absence of a *prima facie* case, appellant's rebuttal evidence was unnecessary:

> We do not find it necessary to reach the question of the weight to be given the papers presented to the New York [**82] Academy of Sciences in that appellant has no *prima facie* showing of obviousness to rebut.

Id. at 702 n.9, 200 USPQ at 719 n.9.

Over the remaining years of the CCPA's existence its opinions were steadfast in requiring consideration of both structure and properties in determinations of *prima facie* obviousness. Precedent had ripened, as exceptions diminished. See *Kotteakos v. United States, 328 U.S. 750, 66 S. Ct. 1239, 90 L. Ed. 1557 (1946)*, wherein the Supreme Court discusses how case by case determination on a variety of facts in time shows the direction of the law:

> For, as with all lines which must be drawn between positive and negative fields of law, the precise border may be indistinct, but case by case determination of particular points adds up in time to discernible direction.

328 U.S. at 761-62.

Further illustrations of the direction established by precedent are, *e.g., In re Swan Wood, 582 F.2d 638, 641, 199 USPQ 137, 139 (CCPA 1978)*:

> In view of the close structural similarity between the claimed compounds and [the prior art compound], and the fact that [**83] the latter is disclosed as possessing anti-microbial activity, we believe that one skilled in the art would have been, *prima facie*, motivated to make the claimed [*713] compounds in the expectation that they, too, would possess antimicrobial activity.

In *In re Payne, 606 F.2d 303, 203 USPQ 245 (CCPA 1979)*, the court stated:

> The similarity in structure and properties between the prior art and claimed compounds is sufficiently close to support a *prima facie* case of obviousness.

Id. at 314, 203 USPQ at 255. The court explained:

> An obviousness rejection based on similarity in chemical structure and function entails the motivation of one skilled in the art to make a claimed compound in the expectation that compounds similar in structure will have similar properties.

Id. at 313, 203 USPQ at 254.

In *In re Grunwell, 609 F.2d 486, 203 USPQ 1055 (CCPA 1979)* the court held that a *prima facie* case of obviousness was made for certain new steroids, and not for others. For compounds where the structures differed from the prior art only in a methyl substituent, [**84] and the physiologic and psychologic properties were similar, a *prima facie* case was deemed made. *Id. at 491, 203 USPQ at 1059.* Other Grunwell steroids were ethers, while the closest structures in the prior art were alcohols; in that case the court held that a *prima facie* case was not made, because the examiner had not shown why one skilled in the art would have replaced this hydroxyl group with an ether. *Id.*

Similar reasoning was applied to process claims in *In re Clemens, 622 F.2d 1029, 206 USPQ 289 (CCPA 1980).* Claims to the use of new polymeric exchange resins to remove corrosion products in a steam system were rejected as *prima facie* obvious from references describing structurally similar polymers used for the same purpose. However, the court found that the PTO did not make a *prima facie* case as to claim 8 because of temperature limitations not shown in the prior art. Therefore:

> Since the PTO had not made out a prima facie case of obviousness respecting claim 8, evidence of comparative testing was unnecessary in rebuttal.

Id. at 1036, 206 USPQ at 296.

Again in *In re Zeidler, 682 F.2d 961, 215 USPQ 490 (CCPA 1982)* [**85] a *prima facie* case was deemed made based on similarity of structure and of properties:

> One of ordinary skill would have had reason to expect, given the close structural similarity of the [reference] compounds and the teachings of [another reference], that use of a sulfonamide bridge . . . would have resulted in dyes possessing the same or only slightly different properties from prior art dyes.

Id. at 966, 215 USPQ at 494. The *prima facie* case was held rebutted as to one claimed dye compound, but not as to another. *Id.*

The Federal Circuit did not stray from this precedent. [16] As summarized in *In re Grabiak, 769 F.2d 729, 731, 226 USPQ 870, 871 (Fed. Cir. 1985)*:

> When chemical compounds have "very close" structural similarities and similar utilities, without more a *prima facie* case may be made.

The rule also continued to be applied to process (use) claims, as illustrated in *In re Merck & Co., 800 F.2d 1091, 231 USPQ 375 (Fed. Cir. 1986)*, wherein the court held that "close structural similarity and a similar use" of the applicant's known compound and the prior art compound [**86] made a *prima facie* case of obviousness of claims to the asserted new use. *Id. at 1097, 231 USPQ at 379.*

16 Although the majority states that it is "not retreating from the recent trend of case law", the cases over the past thirteen years (since *Shetty)* do not support this view. The courts have generally required that there be a suggestion in the prior art that would have made obvious not only the chemical structure but also the newly discovered property of a new chemical compound or composition, in order to make a *prima facie* case under *section 103.*

In *In re Geiger, 815 F.2d 686, 2 USPQ2d 1276 (Fed. Cir. 1987)* the Federal Circuit held that although the prior art disclosed the separate components of the claimed new compositions, for the same general use of treating cooling water systems, a *prima facie* case was not established "absent some teaching, suggestion or incentive supporting [*714] the combination". The court held that:

> Because we [**87] reverse on the basis of failure to establish a *prima facie* case of obviousness, we need not reach the issue of the sufficiency of the showing of unexpected results.

Id. at 688, 2 USPQ2d at 1278.

In *In re Chupp, 816 F.2d 643, 2 USPQ2d 1437 (Fed. Cir. 1987)* the applicant did not challenge the board's holding that a new compound useful as a herbicide was *prima facie* obvious in view of the prior art showing of an adjacent homolog and other structurally similar compounds used as herbicides. However, the court found the rebuttal evidence of differences in selectivity sufficient to hold the composition claims allowable. *Id. at 647, 2 USPQ2d at 1440.*

In *Chupp* the court disposed of the Commissioner's policy argument that grant of the composition claims would prevent the public from using Chupp's structurally obvious compound for the herbicidal uses shown in the prior art, with the remark that "the expectation that persons would want to use the compound to produce inferior results (or would want to fight lawsuits over such uses) is false." *Id. at 647, 2 USPQ2d at 1440.*

The Federal Circuit has [**88] not applied the standard of *prima facie* obviousness that the majority today "reaffirms", but has consistently considered properties and use as well as structure. The only decision referred to by the majority is *In re Wright, 848 F.2d 1216, 6 USPQ2d 1959 (Fed. Cir. 1988)*, as if *Wright* stood alone in its requirement that the inventor's desired properties and use must be considered. *Wright* dealt with a mechanical device, and is in the mainstream of this precedent. In *Wright* we said:

> The determination of whether a novel structure is or is not "obvious" requires cognizance of the properties of that structure and the problem which it solves, viewed in light of the teachings of the prior art.

Id. at 1219, 6 USPQ2d at 1361 (citations omitted). Not one of the myriad cases wherein the courts had required consideration of these factors is mentioned or distinguished by the majority, although presumably *all* are now overruled. In this already-lengthy survey I have not included mechanical cases whose rationale is stated in the same terms as that of *Wright*. [17] Nonetheless the *in banc* court today overrules *Wright* [**89] , without argument or briefing, and extends to mechanical devices the [*715] same theory of structural obviousness that the court now applies to chemicals, discarding the extensive precedent to the contrary.

17 *Wright* claimed a new carpenter's level, having the new property and use of enhanced pitch measurement. Wright's new structure was a combination of elements that were in the prior art, but there was no suggestion in the prior art that this new combination might have the property and use discovered by Wright. The panel explained that it was unobvious to make this combination to solve the problem of increasing pitch measurement: a rationale appearing in dozens of decisions. *See, e.g., Lindemann Maschinenfabrik GmbH v. American Hoist and Derrick Co., 730 F.2d 1452, 1462, 221 USPQ 481, 488 (Fed. Cir. 1984)* ("Nothing in the references alone or together suggests the claimed invention as a solution to the problem of crushing rigidly massive scrap"); *In re Benno, 768 F.2d 1340, 1347, 226 USPQ 683, 687 (Fed. Cir. 1985)* ("[Benno] had to invent a solution to that problem. . . . Neither reference hints at his solution"); *Weather Engineering Corp. of America v. United States, 614 F.2d 281, 287, 204 USPQ 41, 46-7 (Ct. Cl. 1980)* ("The near unanimous approach by the courts is that 'the prior art that is relevant in evaluating a claim of obviousness is defined by the nature of the problem confronting the would-be inventor'"); *In re Naber, 494 F.2d 1405, 1407, 181 USPQ 639, 641 (CCPA 1974)* ("even if one of ordinary skill in the art were moved to combine the references, there would be no recognition that the problem of combustible deposits had been solved"); *In re Aufhauser, 55 C.C.P.A. 1477, 399 F.2d 275, 281, 158 USPQ 351, 355 (CCPA 1968)* ("as in *United States v. Adams, 383 U.S. 39, 86 S. Ct. 708, 15 L. Ed. 2d 572 (1966)*, what appellant had done was to *observe* an existing problem in the art which had not been solved by the prior art and then *combine* individually old concepts to solve that problem") (emphasis in original); *In re Rothermel, 47 C.C.P.A. 866, 276 F.2d 393, 125 USPQ 328, 331 (CCPA 1960)* ("Where the invention for which a patent is sought solves a problem which persisted in the art, we must look to the problem as well as its solution if we are to properly appraise what was done and to evaluate it against what would be obvious to one having the ordinary skills of the art."); *In re Ratti, 46 C.C.P.A. 976, 270 F.2d 810, 813, 123 USPQ 349, 351 (CCPA 1959)* (the prior art did not teach "how to solve the problems" faced by the inventor).

Many other decisions apply similar reasoning, contrary to the majority's holding today.

[**90] The Court stated in *Thomas v. Washington Gas Light Co., 448 U.S. 261, 272, 100 S. Ct. 2647, 65 L. Ed. 2d 757 (1980)*:

> When rights have been created or modified in reliance on established rules of law, the arguments against their change have special force.

At least, the majority should explain its reasoning for so far-reaching a change of law and practice, affecting patentability of mechanical devices as well as chemical compounds and compositions.

D

The Statute

The court's *in banc* holding that similarity of chemical (and mechanical) structure suffices for *prima facie* unpatentability under *35 U.S.C. § 103*, even when the applicant's newly discovered properties and use are not suggested in the prior art, departs from the precepts of not only *section 103*, but also *sections 101* and *102*. The court today imposes the same rebuttal burden on the applicant regardless of whether the prior art suggests the applicant's newly discovered properties.

35 U.S.C. § 103 requires that obviousness be determined in light of the prior art; *section 102* fixes the limits of prior art; and *section 101* requires [**91] utility as a condition of patentability. Giving consideration to the newly discovered properties and utility as well as the structure of a new chemical compound or composition (or a new device) implements the requirement of *section 103* that the invention be viewed as a whole. *Jones v. Hardy, 727 F.2d 1524, 1529, 220 USPQ 1021, 1025 (Fed. Cir. 1984)* ("Failure to consider the claimed invention as a whole is an error of law"); *In re Kuehl, 475 F.2d 658, 664-65, 177 USPQ 250, 255 (CCPA 1973)* ("The test under *§ 103* is whether in view of the prior art the invention as a whole would have been obvious at the time it was made"). As the court remarked in *Lunsford*, it is unlikely that a person of ordinary skill would consider only the structures, and not the properties, described in the prior art.

The factual determination of the scope and content of the prior art, *see Graham*, 383 U.S. at 17, 148 USPQ at 467, is, of course, directed to prior art that meets the conditions of *section 102*. *Section 102* describes prior art as what is published or otherwise known, including subject matter in public use or on sale. Not included is what is [**92] unknown, or knowledge that became known to the inventor through the inventor's own research:

> To rely on an equivalence *known only to the applicant* to establish obviousness is to assume that his disclosure is a part of the prior art. The mere statement of this proposition reveals its fallaciousness.

In re Ruff, 45 C.C.P.A. 1037, 256 F.2d 590, 598, 118 USPQ 340, 347 (CCPA 1958) (emphasis in original). Indeed, this principle was recently modernized by Congress in *35 U.S.C. § 103*, second paragraph (1984) (prior art does not include certain information of common ownership). When the prior art does not suggest that similar structures will have the property and use that the inventor discovered, such fact can not be used to support rejection of the claims, even when the inventor included such information in the patent application.

35 U.S.C. § 101 requires that an invention be useful. The use of a chemical is the utilitarian property of that chemical. Since the essence of the *prima facie* case is that the holding of unpatentability is legally complete, the property and utility can not be ignored. Consideration [**93] of the utility of the claimed invention is as integral to the examiner's determination of *prima facie* unpatentability as it is to the decision after any rebuttal evidence is submitted.

The decisions I have discussed, and many others, illustrate the breadth of the court's exposure and the depth of the court's understanding, evolved over more than thirty years of application of these statutory principles. The weight of precedent, well exceeding the sampling I have reported, contradicts the majority's holding, and has established powerful legal principles that should not be discarded without sound [*716] reason. As discussed by the Supreme Court in *Vasquez v. Hillery, 474 U.S. 254, 265, 106 S. Ct. 617, 88 L. Ed. 2d 598 (1986)*,

> *stare decisis* [is] the means by which we ensure that the law will not merely change erratically, but will develop in a principled and intelligible fashion. That doctrine permits society to presume that bedrock principles are founded in the law rather than in the proclivities of individuals. . . .

The law of *prima facie* obviousness had developed over a long history, knitting the common threads of many carefully [**94] considered cases, leading to unifying criteria, in the tradition of the common law.

The court has made the wrong choice in reviving the *Hass-Henze* presumption of obviousness based on chemical structure without consideration of the obviousness of the applicant's new properties. This presumption was criticized, limited, and overruled, and has been superseded by judicial appreciation that a chemical "is, realistically and legally, a composite of both structure and properties", in the words of *Papesch*. I repeat the unifying criterion that for a new chemical compound or composition a *prima facie* case of obviousness is made when both (1) the new compound or composition is of closely related chemical structure to the prior art compound or composition, and (2) there is some suggestion or motivation arising in the prior art to make the new compound or composition in order to achieve the inventor's desired properties and utility. When these requirements were met, appropriate evidentiary showings could rebut the *prima facie* case. Only an occasional exception among past decisions eliminated the second part of this unifying criterion, and required proof of actual differences in [**95] properties when there was no suggestion in the prior art of the properties discovered by the inventor. Today the aberration becomes the law, reversing over thirty years of reasoned legal analysis.

The court does not state what new statutory interpretation is invoked, what new policy or principle is served, by rejecting the reasoning of so many decisions. The Supreme Court has remarked that

> any detours from the straight path of *stare decisis* in our past have occurred for articulable reasons, and only when the Court has felt obliged "to bring its opinions into agreement with experience and with facts newly ascertained."

Vasquez, 474 U.S. at 266 (quoting Burnet v. Coronado Oil & Gas Co., 285 U.S. 393, 412, 52 S. Ct. 443, 76 L. Ed. 815 (1932) (Brandeis, J., dissenting)). When our court makes so dramatic a change of law, its *ratio decidendi* should be made known.

II

DILLON'S INVENTION

Dillon's patent application discloses and claims her discovery that the inclusion of certain tetra-orthoester compounds in hydrocarbon fuel compositions will reduce the emission of solid particulates (*i.e.* soot) during combustion of [**96] the fuel. The tetra-orthoesters are a known class of chemical compound. It is undisputed that their combination with hydrocarbon fuels, for any purpose, is not described in the prior art; nor is their use to reduce particulate emissions from combustion of hydrocarbon fuels.

A

Dillon's Appeal to the Board

An applicant is required to appeal from all of the examiner's rejections that the applicant wishes to contest. *37 C.F.R. § 1.191(c)*. Dillon did so, and appealed the rejection of claims 2-14, 16-22, and 24-37. Of these, claims 24-35 were process (use) claims and the others were composition claims. The appealed rejections were directed to the process and composition claims together, and were described by the board as follows:

> 1. Claims 24 through 37 under *35 U.S.C. 112*, second paragraph.
>
> 2. Claims 2 through 14, 22 and 34 through 37 under *35 U.S.C. 103* as unpatentable over Sweeney '417 in view of Elliott, Howk, Kesslin, Speh, and Neves. [*717]
>
> 3. Claims 16 through 22, 24 through 33, 35, 36 and 37 under *35 U.S.C. 103* as unpatentable over Sweeney '267 or '417 in view of Elliott, Howk, [**97] Kesslin and Speh.

Board op. at 2. In its decision the board wrote that composition and method of use claims were at issue:

> The appealed subject matter relates to compositions comprising a hydrocarbon fuel and a particulate emissions suppressing amount of an organic orthoester, and a method of reducing particulate emissions by combusting such compositions.

Board op. at 1.

Dillon duly argued each ground of rejection, in accordance with *37 C.F.R. § 1.192*, and the examiner responded. The board reversed the examiner's rejection on *section 112*, for all the claims rejected on that ground, and affirmed the rejections on *section 103*, for all the claims rejected on that ground. Each ground encompassed composition and process claims, without distinction. (The board also discussed claims 13, 14, 34, 36, and 37, as to aspects not here material.)

Analyzing all the claims with respect to *section 103*, the board did not distinguish among them. For example, the board stated that

> differences between appellant's and the prior art's motivation for adding a component to a composition will not alone render the claimed *composition, or process* unobvious.

[**98] Board op. at 7 (emphasis added). The board further stated, again as to all the claims, that

> The mere recitation of a newly discovered function inherently possessed by *things and processes* in the prior art does not cause claims drawn thereto to distinguish over that prior art.

Board op. at 8 (emphasis added). Throughout its opinion the board did not imply that its analysis was directed solely to the composition claims. Indeed, the board stated that

> the *use* of the orthoesters recited in the appealed claims (wherein R is -OR) as hydrocarbon fuel additives would clearly have been *prima facie* obvious from the teachings of the Sweeney patents alone and the close chemical and structural relationship between Sweeney's and appellant's orthoesters[.]

Board op. at 6 (emphasis added). The majority errs in its statement that the board "reviewed only the composition claims". The board considered and rejected all the claims, drawing no distinction as to the style of the claim. [18]

> 18 The process claims were written in the style required by *35 U.S.C. § 100(b)*, *see* n.15, *supra*. As explained in *In re Moreton, 48 C.C.P.A. 875, 288 F.2d 708, 709, 129 USPQ 227, 228 (CCPA 1961)*, "this mere matter of form [i.e., claiming a new use as a process] should have no effect on patentability".

[**99] The rejection of all of the claims was appealed to us. The Commissioner has not argued otherwise, and both sides briefed and argued all the claims. As stated in the Commissioner's brief, the issue on appeal is whether "The claimed subject matter would have been *prima facie* obvious from the combined teachings of the references." Thus I must dissent from the court's affirmance of the board's rejection of the process claims simply because Dillon did not argue that the process claims are patentable even if the composition claims are not.

B

In re Durden

Before this court, Dillon properly did not discuss points that had not been raised by the examiner or the board. Since there had been no reliance on the law of *In re Durden, 763 F.2d 1406, 226 USPQ 359 (Fed. Cir. 1985)*, and neither the examiner nor the board cited *Durden*, Dillon did not discuss the law of *Durden*.

The Commissioner, in his answering brief before the Federal Circuit, argued for the first time that *Durden* provided additional authority for upholding the board's rejection of the process claims. The Commissioner stated that if the court decided to [*718] reverse the board's holding [**100] of unpatentability, the court should consider the effect of *Durden*. The panel, having reversed the board's holding, thus considered *Durden*, as the Commissioner requested; the panel simply observed that *Durden* does not apply to "use" claims.

However, the majority of this court, having affirmed the rejection of all the claims, has no basis for review on additional authority. Thus I can not join the majority's opinion interpreting *Durden*, for it is, in the event, *dictum*.

C

The Merits

Applying the guidance of precedent to Dillon's invention: the compositions are new, [19] and their property and use of reducing particulate emissions is not taught or suggested in the prior art. There is no objective teaching in the prior art that would have led one of ordinary skill to make this product in order to solve the problem that was confronting Dillon: to reduce soot from combustion of hydrocarbon fuels. There is no reasonable basis in the prior art for expecting that Dillon's new compositions would have the particulate-reducing property that she discovered. As shown in Part I, *ante*, structure, properties and use must be considered in determining whether a *prima* [**101] *facie* case under *section 103* has been made.

> 19 If a compound or composition is known, for any use or no use, it is not patentable. *35 U.S.C. § 102*; *Titanium Metals Corp. v. Banner, 778 F.2d 775, 780, 227 USPQ 773, 777-78 (Fed. Cir. 1985)*.

The Sweeney references show the water-sequestration property of tri-orthoesters in hydrocarbon fuels, and the Elliott reference shows the water-sequestration property of tri- and tetra-orthoesters in hy-

draulic fluids (which are not hydrocarbons and not fuels). There is no suggestion in the prior art that would have led one of ordinary skill to make Dillon's new compositions in the expectation that they would reduce particulate emissions from combustion. No reference suggests any relationship between the properties of water-sequestration and soot-reduction. All this is undisputed.

Dillon raises the question of whether the Sweeney and Elliott references [20] are properly combinable, arguing that they are not in analogous arts. [**102] This question need not be decided, for even when combined these references offer no suggestion of the property of reducing particulate emissions from combustion. *In re Naber, 494 F.2d 1405, 1407, 181 USPQ 639, 641 (CCPA 1974)* ("even if one of ordinary skill in the art were moved to combine the references, there would be no recognition that the problem of combustible deposits had been solved").

20 The Board held that all the references other than Sweeney and Elliott were "merely cumulative", and did not discuss the Howk reference, on which the majority apparently now relies.

The board stated that it is inherent in Dillon's compositions that they would reduce particulate emissions, that Dillon "merely recited a newly discovered function inherently possessed" by the prior art. Arguments based on "inherent" properties can not stand when there is no supporting teaching in the prior art. Inherency and obviousness are distinct concepts. *In re Spormann, 53 C.C.P.A. 1375, 363 F.2d 444, 448, 150 USPQ 449, 452 (CCPA 1966)*: [**103]

> The inherency of an advantage and its obviousness are entirely different questions. That which may be inherent is not necessarily known. Obviousness cannot be predicated on what is unknown.

When the PTO asserts that there is an explicit or implicit teaching or suggestion in the prior art, the PTO must produce supporting references. *In re Yates, 663 F.2d 1054, 1057, 211 USPQ 1149, 1151 (CCPA 1981).*

The applicant's newly discovered properties must be considered in determining whether a *prima facie* case of unpatentability is made, along with all the other evidence. Neither structure nor properties can be ignored; they are essential to consideration of the invention as a whole. But Dillon's own discovery of the soot-reducing [*719] property of the tri-orthoester fuel composition is not evidence against her in determining whether the prior art makes a case of *prima facie* obviousness. *In re Wertheim, 541 F.2d 257, 269, 191 USPQ 90, 102 (CCPA 1976)* (applicant's own disclosures can not be used to support a rejection of the claims "absent some admission that matter disclosed in the specification is in the prior art"); [**104] *In re Ruff, 256 F.2d at 598, 118 USPQ at 347* ("The mere statement of this proposition reveals its fallaciousness").

The board cited *In re Merck, 800 F.2d at 1097, 231 USPQ at 379*, in arguing that obviousness does not require absolute predictability. Obviousness does, however, require a sufficient relationship between the use taught in the reference and the use discovered by the applicant. In *Merck* the reference compound and the claim compound were both known to have psychotropic properties, supporting the holding of *prima facie* obviousness of the claimed specific antidepressant use. Applying this reasoning to Dillon's claims leads to the opposite conclusion, for Dillon's use to reduce soot from combustion is not suggested by the known use of the prior art compositions to scavenge water. (Only "use" claims were present in *Merck* -- again illustrating that the board, citing *Merck* against Dillon's claims, did not distinguish between composition and use claims in its analysis of Dillon's invention.)

In view of the complete absence of any suggestion in the prior art that Dillon's new compositions would have her newly discovered and unobvious [**105] property and use of soot reduction, I would reverse the rejection of the composition and the use claims.

The Commissioner raised the policy argument that Dillon is simply removing from the public an obvious variant of Sweeney's and Elliott's compositions, one that might be useful to scavenge water in fuels. In *Ruschig* the court had considered the argument, and remarked that the provision of adequate patent protection for the applicant's new compounds, not previously in existence and having a new and unobvious use, was favored over the "mere possibility that someone might wish to use some of them for some such [other] purpose". *343 F.2d at 979, 145 USPQ at 286*. See also, e.g., *Chupp, 816 F.2d at 647, 2 USPQ2d at 1440*, wherein the court expressed a similar view. This practical wisdom has been tested by long experience. It accords with judicial recognition that:

> Although there is a vast amount of knowledge about general relationships in the chemical arts, chemistry is still largely empirical, and there is often great difficulty in predicting precisely how a given compound will behave.

In re Carleton, 599 F.2d 1021, 1026, 202 USPQ 165, 170 (CCPA 1979). [**106]

Granting Dillon a patent on her invention takes away nothing that the public already has; and the public receives not only the knowledge of Dillon's discovery, for abandoned patent applications are maintained in secrecy, but Dillon is not deprived of an incentive to discover and to commercialize this new product for this new use. [21]

> 21 The majority remarks that Dillon made "no attempt to argue the relative importance" of soot reduction and water sequestration. This dramatic new criterion, although presented in the *amicus* brief of the American Intellectual Property Law Association, should not be approved by this *in banc* court without discussion.

Conclusion

Following the weight of precedent, I would hold that a *prima facie* case of obviousness of a new chemical compound or composition requires consideration of not only the chemical structure but also the newly discovered properties, in light of the teachings and suggestions of the prior art. I would expressly reject the Commissioner's position [**107] that determination of the *prima facie* case is made regardless of the properties disclosed in the inventor's application.

Since there is no suggestion in the prior art references, alone or in combination, of the particulate-reducing property and use discovered by Dillon for her new compositions, a *prima facie* case of obviousness has not been made. Thus it is not necessary to patentability that Dillon establish [*720] that the prior art compositions do not possess the same soot-reduction property and use. I would reverse the board's rejection of claims 2-14, 16-22, and 24-37, all of the claims before us on appeal.

110TVC

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U.S. Appln. No. 10/770,639
Reference No. 23

LEXSEE 769 F.2D 729

In re RAYMOND C. GRABIAK, et al.

No. 84-1718

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

769 F.2d 729; 1985 U.S. App. LEXIS 15062; 226 U.S.P.Q. (BNA) 870

August 9, 1985, Decided

PRIOR HISTORY: [**1] Appealed from: U.S. Patent & Trademark Office Board of Appeals.

DISPOSITION: Reversed.

CASE SUMMARY:

PROCEDURAL POSTURE: Appellant challenged a decision of the Patent and Trademark Office Board of Appeals sustaining a rejection of its claims in its patent application.

OVERVIEW: Appellant's patent application was rejected by the Patent and Trademark Office as unpatentable under *35 U.S.C.S. § 103*. This conclusion was affirmed, and appellant challenged in federal court. In reversing, the federal court of appeals concluded that the Patent and Trademark Office had failed to establish a prima facie case of obviousness in the absence of adequate support in the prior art. Where no prima facie case of obviousness was established, the burden of proof did not shift to appellant. Reversal was thus warranted.

OUTCOME: The decision of the Patent and Trademark Office Board of Appeals was reversed, as the Patent and Trademark Office had failed to establish a prima facie case of obviousness; thus, the burden of proof had not shifted to appellant.

LexisNexis(R) Headnotes

Patent Law > Nonobviousness > Elements & Tests > General Overview
Patent Law > Utility Requirement > Chemical Compounds
[HN1] When chemical compounds have "very close" structural similarities and similar utilities, without more, a prima facie case may be made. When such "close" structural similarity to prior art compounds is shown, the burden of coming forward shifts to the applicant, and evidence affirmatively supporting unobviousness is required.

Patent Law > Nonobviousness > Elements & Tests > General Overview
Patent Law > Utility Requirement > Chemical Compounds
[HN2] The mere fact that it is possible to find two isolated disclosures that might be combined in such a way to produce a new compound does not necessarily render such production obvious unless the art also contains something to suggest the desirability of the proposed combination.

COUNSEL: J. Timothy Keane, Monsanto Company, of St. Louis, Missouri, argued for Appellants.

Fred W. Sherling, United States Patent & Trademark Office, of Arlington, Virginia, argued for Appellee. With him on the brief were Joseph F. Nakamura, Solicitor, Joseph W. Dewhirst, Associate Solicitor, and Harris A. Pitlick, Associate Solicitor.

JUDGES: Friedman, Nies, and Newman, Circuit Judges.

OPINION BY: NEWMAN

OPINION

[*729] NEWMAN, Circuit Judge.

Raymond C. Grabiak *et al.* appeal from the decision of the Patent and Trademark Office Board of Appeals sustaining the rejection of claims 1 through 34, all of the claims of patent application Serial No. 168,959, filed July 17, 1980 for "2-Chloro-4-Trifluoromethyl Thiazolecarbothioic Acids Useful As Herbicidal Safeners", as

unpatentable under *35 U.S.C. § 103*. We conclude that the PTO has not presented a *prima facie* case of unpatentability, and on this basis we reverse the decision of the Board.

The Invention

The claimed invention relates to a class of chemical compounds having utility as herbicidal safeners. Safeners, [**2] sometimes called antidotes, are used to protect growing crops from damage that may be caused by the application of herbicides to control undesired plants. The claimed compounds, useful as safeners against acetanilide herbicides, are certain thiazole thiocarboxylates [*730] as shown in Claim 1, the broadest claim:

[SEE ILLUSTRATION IN ORIGINAL]

1. A compound of the formula wherein R is C1-5alkyl, phenyl or benzyl. Other claims are directed to various species, to herbicidal mixtures containing these compounds, and to various methods of use of these compounds. Grabiak has not argued the claims separately, and we do not so consider them.

The Rejection

The claims stand rejected as obvious from Howe *et al.* U.S. Patent No. 4,199,506. Also relied on are Bollinger U.S. Patent No. 4,317,310 and R. Conant & A. Blatt, *The Chemistry of Organic Compounds* 342-43 (3d ed. 1947), an organic chemistry textbook.

Howe describes a family of chemical compounds having utility as safeners for acetanilide herbicides, consisting of thiazole caboxylic and thiazole carboxamide compounds of the general formula:

[SEE ILLUSTRATION IN ORIGINAL]

In the Howe disclosure [**3] R, R', n, and X, are broadly defined, the breadth of which is not pertinent to this issue. Very pertinent is the disclosure in Howe of the following specific compound:

[SEE ILLUSTRATION IN ORIGINAL]

This compound differs from those claimed by Grabiak only by the presence in Grabiak of a sulfur atom instead of a particular oxygen atom in the ester moiety, a difference which the examiner asserted would have been, without more, obvious.

The examiner cited the Bollinger reference as showing the interchangeability of oxygen and sulfur in compounds having safening properties. Bollinger shows, as safeners for thiocarbamate and acetanilide herbicides, a class of 2-imino derivatives of 1,3-oxathioles and 1,3-dithioles. The examiner pointed to the 1,3-oxathiole/dithiole ring fragment:

[SEE ILLUSTRATION IN ORIGINAL]

wherein Z is defined as either oxygen or sulfur, as support for the conclusion that it would have been obvious to exchange a sulfur atom for an oxygen atom in the Howe compounds. The Board agreed.

[*731] On reconsideration, the Board in a split decision affirmed the rejection, citing *In re Fancher, 56 C.C.P.A. 1121, 410 F.2d 813, 161 U.S.P.Q. (BNA) 613 (CCPA 1969)* [**4] and *In re Albrecht, 579 F.2d 92, 198 U.S.P.Q. (BNA) 208 (CCPA 1978)* for the proposition that oxygen and sulfur are well known to be interchangeable. To "reiterate that the close analogy between sulfur and oxygen isologs is well known," the Board referred to Conant & Blatt's discussion of the general similarities between simple sulfur and oxygen compounds. One member of the Board dissented, stating his belief that the compounds disclosed in Bollinger are "too remote to those claimed" to suggest substitution of sulfur for oxygen at a particular place in the Howe compounds.

The Argument

Grabiak presented no evidence that his safener compounds have unobvious properties as compared with Howe's safener compounds, and stated plainly that they do not. Grabiak's argument is, in sum, that (1) in the field of biological activity, it is not predictable whether chemical compounds that have an apparent structural similarity will also have similar biological properties; (2) biological properties cannot be predicted; they must be determined by experimentation; (3) therefore mere structural similarity is inadequate to present a *prima facie* case of obviousness; and [**5] (4) more is required, such as suggestion in the prior art (a) that the structural modification should be made and (b) that the modified compound will exhibit the biological behavior of the prior art compound.

Grabiak argues that Howe does not teach that one of the oxygens in the Howe carboxylate group could be replaced with sulfur to produce safeners for acetanilide herbicides, and that Bollinger and Conant & Blatt do not cure this deficiency because Bollinger is dealing with a quite different part of a quite different molecule, and the Conant & Blatt text refers only to simple structures and chemical, not biological, properties; and in any event that safening activity is, like all biological behavior, unpredictable. Grabiak asserts that the teachings of Howe with Bollinger and Conant & Blatt are insufficient to establish *prima facie* obviousness, in that there is no motive in the cited art to make the modification required to arrive at appellants' compounds.

Analysis

I.

[HN1] When chemical compounds have "very close" structural similarities and similar utilities, without more a [**6] *prima facie* case may be made. See for example *In re Wilder, 563 F.2d 457, 195 U.S.P.Q. (BNA) 426 (CCPA 1977)* (adjacent homologues and structural isomers); *In re May, 574 F.2d 1082, 197 U.S.P.Q. (BNA) 601 (CCPA 1978)* (steroisomers); *In re Hoch, 57 C.C.P.A. 1292, 428 F.2d 1341, 166 U.S.P.Q. (BNA) 406 (CCPA 1970)* (acid and ethyl ester). When such "close" structural similarity to prior art compounds is shown, in accordance with these precedents the burden of coming forward shifts to the applicant, and evidence affirmatively supporting unobviousness is required.

Analysis of those circumstances in which a *prima facie* case has or has not been made in view of the degree of structural similarity or dissimilarity, or the presence or absence of similar utility between the prior art compound and that of the applicant, has inspired generations of applicants, courts, and scholars. Upon review of this history, we have concluded that generalization should be avoided insofar as specific chemical structures are alleged to be *prima facie* obvious one from the other. Although we do not accept Grabiak's argument that when biological [**7] activity is involved there can be no presumption (i.e. no *prima facie* case) of obviousness, in the case before us there must be adequate [*732] support in the prior art for the ester/thioester change in structure, in order to complete the PTO's *prima facie* case and shift the burden of going forward to the applicant.

The Bollinger teaching of various heterocyclic rings containing either two sulfur atoms or one oxygen and one sulfur atom, rings which are unlike any part of the Howe molecule, does not suggest the interchangeability of sulfur for oxygen in the ester moiety of the Howe molecule. (Grabiak also analyzes the Bollinger disclosure as showing "dramatic decreases in safener activity when replacing oxygen with sulfur".) Conant & Blatt's brief discussion that "simple sulfur compounds" have properties similar to simple oxygen compounds does not purport to apply to complex organic molecules. Nor do the *Fancher* and *Albrecht* cases remedy these deficiencies, for in each of those cases the sulfur/oxygen interchange was in a heterocyclic ring common to both the prior art compounds and the applicant's compounds.

We repeat the statement of *In re Bergel, 48 C.C.P.A. 1102, 292 F.2d 955, 956-57, 130 U.S.P.Q. (BNA) 206, 208 (CCPA 1961)*, [**8] that:

> [HN2] The mere fact that it is *possible* to find two isolated disclosures which might be combined in such a way to produce a new compound does not necessarily render such production obvious unless the art also contains something to suggest the desirability of the proposed combination. [Emphasis in original]

The PTO cited no pertinent reference showing or suggesting to one of ordinary skill in the art the change of a thioester for an ester group. In the absence of such reference, there is inadequate support for the PTO's position that this modification would *prima facie* have been obvious.

II.

The Solicitor contends that the sulfur in Grabiak that replaced the oxygen in Howe occurs in a portion of the molecule that is not significant to safener activity, as further argument that Grabiak's compounds would have been obvious from Howe's compounds. To support this argument the Solicitor refers to the statement in Howe that the carboxylic moiety may include the acid and salts thereof, acid chlorides, amides, and esters. From this the Solicitor argues that the nature [**9] of this moiety "would not be expected to impart or contribute to the safening utility", and therefore that the replacement of Howe's ester with Grabiak's thioester would have been obvious.

This argument is lacking in a critical element: adequate support in the prior art. Howe does not state that the carboxylic segment of his molecule is not significant to its biological properties, and no other support is invoked. We appreciate that the PTO lacks the possibility of experimental verification of this theory; but absent an initial *prima facie* case, we do not think the burden of disproving this theory is shifted to Grabiak. Nor do we judicially accept a theory that appears to require the general assumption that sulfur is not significant to biological behavior.

Grabiak argues further that the PTO's position that the identity of the carboxylic component is not material cannot apply here because safening activity can not be predicted from chemical structure. Grabiak asserts that the efficacy of any compound for safening depends on variables including the type of herbicide compound, the type of weed to be controlled, the type of crop to be protected and the safener compound itself. [**10] Grabiak cites data from Howe which he states show that a "compound, which safens one herbicide used to control barnyard grass in the presence of corn crop, is totally ineffective to safen that same herbicide to control barnyard grass in the presence of rice." Grabiak also cites data from Bollinger to support Grabiak's position that "safening activity even for closely similar homologues does not vary predictably."

[*733] In response, the Solicitor argues that it is not "necessarily true" that safening activity is not predictable from the structure of the compound. Evidence for this statement is seen by the Solicitor in Grabiak's compounds themselves, which are admitted to have the same safening activity as those of Howe. However, Grabiak's disclosure may not be used to fill the gaps in the prior art. If evidence of similar biological properties between -C(O)OR and -C(O)SR groups is to be relied upon, it must come from the prior art. The PTO produced no such evidence. Instead, the Board held that "it is not inconceivable to substitute [sulfur for oxygen] to obtain compounds having the same expected properties." We agree that it is not inconceivable. The standard, however, [**11] is whether it would have been obvious in terms of *section 103*.

In the absence of adequate support, we conclude that this argument does not perfect the PTO's *prima facie* case.

III.

We have considered the decisions on which the PTO relies. In *In re Payne, 606 F.2d 303, 203 U.S.P.Q. (BNA) 245 (CCPA 1979)* there was prior art well supporting the PTO's *prima facie* case. In *In re Susi, 58 C.C.P.A. 1074, 440 F.2d 442, 169 U.S.P.Q. (BNA) 423 (CCPA 1971)* the difference from the prior art compound was a hydroxyl group, a difference that the applicant conceded was "of little importance". In *In re Doebel, 59 C.C.P.A. 1079, 461 F.2d 823, 174 U.S.P.Q. (BNA) 158 (CCPA 1972)*, the court stated that "the claimed compound is a homologue", and a *prima facie* case was held to have been made. None of these cases requires the result that a thioester derivative be deemed *prima facie* obvious from the corresponding ester in the absence of prior art on this point.

Conclusion

On the record before us, we conclude that the PTO did not establish a *prima facie* case of obviousness, and thus did not shift to Grabiak the burden [**12] of coming forward with evidence of unexpected results.

REVERSED

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U.S. Appln. No. 10/770,639
Reference No. 24

LEXSEE 747 F.2D 703

IN RE JEAN PIERRE LALU and LOUIS FOULLETIER

No. 83-1358

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

747 F.2d 703; *1984 U.S. App. LEXIS 15216*; *223 U.S.P.Q. (BNA) 1257*

November 2, 1984

PRIOR HISTORY: [**1] Appealed from: U.S. Patent and Trademark Office Board of Appeals.

CASE SUMMARY:

PROCEDURAL POSTURE: Appellants sought review of an order of the United States Patent and Trademark Office Board of Appeals, which rejected appellants' patent application for obviousness under *35 U.S.C.S. § 103*.

OVERVIEW: Appellants applied for a patent for an invention relating to a new compound. The United States Patent and Trademark Office Board of Appeals (board) affirmed a patent examiner's rejection of appellants' application based on structural obviousness under *35 U.S.C.S. § 103*. On appeal, the court reversed, since the board erred in its conclusion of prima facie obviousness. In determining whether a case of prima facie obviousness exists, it was necessary to determine whether the prior art teachings would appear to be sufficient to one of ordinary skill in the art to suggest making the claimed substitution or modification. The prior art must provide one of ordinary skill in the art the motivation to make the proposed molecular modifications needed to arrive at the claimed compound. The board failed to properly establish that the claimed compounds would have been prima facie obvious.

OUTCOME: The court reversed the rejection of appellants' patent application on grounds of obviousness, since the board failed to properly establish that the claimed compounds would have been prima facie obvious.

LexisNexis(R) Headnotes

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN1] In determining whether a case of prima facie obviousness exists, it is necessary to ascertain whether the prior art teachings would appear to be sufficient to one of ordinary skill in the art to suggest making the claimed substitution or other modification. The prior art must provide one of ordinary skill in the art the motivation to make the proposed molecular modifications needed to arrive at the claimed compound.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN2] How can there be obviousness of structure, or particularly of the subject matter as a whole, when no apparent purpose or result is to be achieved, no reason or motivation to be satisfied, upon modifying the reference compounds' structure? Where the prior art reference neither discloses nor suggests a utility for certain described compounds, why should it be said that a reference makes obvious to one of ordinary skill in the art an isomer, homolog or analog of related structure, when that mythical, but intensely practical, person knows of no "practical" reason to make the reference compounds, much less any structurally related compounds?

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN3] An element in determining obviousness of a new chemical compound is the motivation of one having ordinary skill in the art to make it. The motivation is not abstract, but practical, and is always related to the properties or uses one skilled in the art would expect the compound to have, if made.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN4] In obviousness rejections based on close similarity in chemical structure, the necessary motivation to

make a claimed compound, and thus the prima facie case of obviousness, rises from the expectation that compounds similar in structure will have similar properties. No common-properties presumption rises from the mere occurrence of a claimed compound at an intermediate point in a conventional reaction yielding a specifically named prior art compound. That an intermediate /end-product relationship exists between a claimed compound and a prior art compound does not alone create a common-properties presumption. Absent that presumption or other evidence of motivation, it cannot be said that it would have been obvious to stop the process for synthesizing the disclosed end product and isolate the claimed intermediate.

Patent Law > Nonobviousness > Elements & Tests > General Overview
[HN5] The court always looks to the subject matter as a whole in determining whether the subject matter would have been obvious at the time the invention was made. Further, a relevant property of a compound cannot be ignored in the determination of non-obviousness.

COUNSEL: Brian M. Poissant, of New York, New York, submitted for the Appellant.

Clyde C. Metzger, of New York, New York, of Counsel.

Joseph F. Nakamura, Solicitor, Jere W. Sears, Deputy Solicitor, and Henry W. Tarring, II, Associate Solicitor, of Arlington, Virginia, submitted for Appellee.

JUDGES: Baldwin, Circuit Judge, Cowen, Senior Circuit Judge, and Kashiwa, Circuit Judge.

OPINION BY: BALDWIN

OPINION

[*703] BALDWIN, Circuit Judge.

This appeal is from a decision of the United States Patent and Trademark Office Board of Appeals (board) affirming the rejection under *35 U.S.C. § 103* of claims 13-22, all of the claims of appellants' application Serial No. 966,508, filed December 4, 1978, for "New Polyfluorinated Sulphonic Acids And Their Derivatives". We reverse.

The Invention

The invention relates to perfluoroalkyl sulfonyl chlorides and bromides having the formula:

C[n]F[2n+1](CH[2])[b]SO[2]Z

wherein the perfluoroalkyl group C[n]F[2n+1] is defined by n being a number between 1 and 20, Z is a chlorine or bromine atom, and the bridging group (CH[2])[b] is defined by b being a number [**2] between 2 and 20.

The claimed compounds are useful in the textile, leather, and paper industries. The compounds have utility as corrosion inhibiting agents, surface active agents, and leveling agents, and therefore can be incorporated into waxes, greases, varnishes, and paints to improve the spreading out and leveling of such viscous products.

Claim 13, the only independent claim on appeal, is illustrative:

> 13. A product having the formula C[n]F[2n+1](CH[2])[b] SO[2]Z wherein C[n]F [2n+1] represents a straight or branched perfluorinated hydrocarbon chain, n is a number between 1 and 20, b is a number [*704] between 2 and 20 and Z is a chlorine or bromine atom.

Claims 14-22 depend from claim 13 and further limit the parameters n, b, and Z which define the length of the perfluoroalkyl group, the length of the bridging group, and the nature of the Z halide group, i.e., a chlorine or bromine atom.

The Prior Art

The sole reference relied upon by the board is United States Patent No. 3,130,221 issued April 21, 1964 to Oesterling. Oesterling discloses 1,1-dihydroperfluoroalkyl sulfonic acids having the formula:

C[n]F[2n+1]CH[2] SO[3]H

[**3] Wherein C[n]F[2n+1] is a lower perfluoroalkyl group and the bridging group is a methylene (CH[2]) group. According to Oesterling, "The compounds of this invention include the 1,1-dihydroperfluoroalkyl acids containing from two to five carbon atoms; i.e., from one to four carbon atoms in the * * * [perfluoroalkyl] portion of the molecule." These compounds are strong acids and are used in reactions such as base neutralization, alkylation catalysis, and metal cleaning. Additionally, the compounds are useful as high energy fuels such as liquid rocket propellants because of their relatively high thermal stability. Of the group of acids disclosed by Oesterling, "the preferred compound for use as a high energy fuel is 1,1-dihydroperfluoroethylsulfonic acid [CF[3]CH[2]SO[3]H]. As the number of carbon atoms in the molecule increases, the thermal stability decreases and compounds containing above five carbon atoms are of little value as a fuel."

The claimed sulfonic acids are prepared in the reference by chlorination of the corresponding bis (1,1-dihydroperfluoroalkyl) disulfides to form the corresponding 1,1-dihydroperfluoroalkyl sulfonyl chlorides, which are then hydrolyzed [**4] to produce the product 1,1-dihydroperfluoroalkyl sulfonic acids. The intermediate sulfonyl chlorides which are used to prepare the final product sulfonic acids have the formula: C[n]F[2n+1]CH[2]SO[2]C1

wherein C[n]F[2n+1] is also a lower perfluoroalkyl group and the bridging group is a methylene (CH[2]) group. Oesterling teaches that the hydrolysis may be carried out without isolation of the intermediate sulfonyl chloride, but it is preferable to hydrolyze isolated sulfonyl chloride in order to obtain a purer sulfonic acid product.

The Rejection

The examiner rejected the claims based on structural obviousness because Oesterling teaches homologous compounds. The examiner said, "Oesterling discloses only one method of preparing the sulfonic acids which *requires* the use of the halide intermediate. One motivated to prepare the homologous acids would similarly be motivated to prepare the homologous acids halides." (emphasis in original).

The board, in affirming the examiner's rejection, said the close structural similarity between the reference sulfonyl chloride compounds and the claimed compounds was sufficient to raise the presumption of obviousness. [**5] The board said further:

> The fact that the reference teaches that the sulfonyl chloride compounds are useful as an intermediate or a starting compound for the production of a corresponding sulfonic acid as opposed to the appellants' disclosure that the claimed compounds have other utilities does not by itself rebut the prima facie case of obviousness made out by the Examiner. * * * The case of *In re Stemniski, 58 C.C.P.A. 1410, 444 F.2d 581, 170 U.S.P.Q. (BNA) 343 (1971)*, is distinguishable since here Oesterling discloses a utility (a starting material for making an acid) for the pertinent sulfonyl chlorides, whereas in *Stemniski* the reference disclosed no utility for the relevant compound. In view of the unequivocal identification and isolation of the sulfonyl chloride by Oesterling and the specific utility taught for the compound, a starting material for the preparation of a useful acid, the portions of the court's decision in *In re Gyurik, 596 F.2d 1012, 201 U.S.P.Q. (BNA) 552 (CCPA* [*705] *1979)*, relied upon by the appellants are not considered to dictate reversal of the Examiner's holding.

OPINION

Appellants argue that the acid taught by Oesterling [**6] is limited to a maximum of five carbon atoms and, therefore, there would be no motivation for one of ordinary skill to prepare an acid, or its predecessor sulfonyl chloride containing more than five carbon atoms. Accordingly, appellants contend that since their compounds may contain up to forty carbon atoms, they are not structurally similar to the Oesterling intermediate sulfonyl chlorides. We disagree with appellants' contentions because the Oesterling teachings regarding the five carbon atom limitation are related only to the use of the product acid as a high energy fuel. Oesterling discloses other uses for the disclosed sulfonic acids, such as in base neutralization, alkylation catalysis, and metal cleaning, to which the teachings of a five carbon atom limitation do not necessarily apply. Moreover, even if the compounds disclosed by Oesterling are limited to compounds containing two to five carbon atoms, the appellants' compounds contain as few as three carbon atoms.

We are, however, persuaded that the board erred in its conclusion of prima facie obviousness.

[HN1] In determining whether a case of prima facie obviousness exists, it is necessary to ascertain whether the prior [**7] art teachings would appear to be sufficient to one of ordinary skill in the art to suggest making the claimed substitution or other modification. *In re Taborsky, 502 F.2d 775, 780, 183 U.S.P.Q. (BNA) 50, 55 (CCPA 1974)*. The prior art must provide one of ordinary skill in the art the motivation to make the proposed molecular modifications needed to arrive at the claimed compound. *In re Stemniski, 58 C.C.P.A. 1410, 444 F.2d 581, 586, 170 U.S.P.Q. (BNA) 343, 347 (CCPA 1971), Taborsky, 502 F.2d at 781, 183 U.S.P.Q. at 55, In re Murch, 59 C.C.P.A. 1277, 464 F.2d 1051, 175 U.S.P.Q. 89 (1972), In re Fay, 52 C.C.P.A. 1483, 347 F.2d 597, 146 U.S.P.Q. (BNA) 47 (1965)*.

In *Stemniski*, the claimed compounds were rejected over structurally closely related compounds disclosed in prior art references. The references did not disclose or suggest any usefulness or significant properties, whereas the applicant disclosed a use for the claimed compounds in his application.

In such a case the court reasoned that the requisite motivation to make the claimed compounds would not be

present. The court doubted whether a prima facie case of obviousness existed:

> [HN2] How can there [**8] be obviousness of structure, or particularly of the subject matter as a whole, when no apparent purpose or result is to be achieved, no reason or motivation to be satisfied, upon modifying the reference compounds' structure? Where the prior art reference neither discloses nor suggests a utility for certain described compounds, why should it be said that a reference makes obvious to one of ordinary skill in the art an isomer, homolog or analog of related structure, when that mythical, but intensely practical, person knows of no "practical" reason to make the reference compounds, much less any structurally related compounds?

444 F.2d at 586, 170 U.S.P.Q. at 347.

Appellants argue that since several utilities were disclosed for the compounds claimed, and Oesterling teaches no significant properties or utility for the disclosed sulfonyl chlorides except as intermediates in the formation of the product sulfonic acids, the rejection of the instant claims is not proper in view of *Stemniski*. The Patent and Trademark Office (PTO) contends that *Stemniski* is satisfied and the rejection is proper because Oesterling discloses that the sulfonyl chlorides are used as intermediates [**9] or starting materials for producing useful acids.

The PTO further argues that the disclosed utility for the Oesterling sulfonyl chlorides as an intermediate for producing useful acids is a usefulness conforming with statutory guidelines, but cites cases [*706] for support which are actually inapposite: *Reiners v. Mehltretter, 43 C.C.P.A. 1019, 236 F.2d 418, 421-22, 111 U.S.P.Q. (BNA) 97, 100 (1956)*, an interference in which structural obviousness was not an issue, and *In re Kirk, 54 C.C.P.A. 1119, 376 F.2d 936, 943-44, 153 U.S.P.Q. (BNA) 266 (1967)*, a case dealing with appellants' disclosure of "how to use" the claimed compounds under *35 U.S.C. § 112.*

Other cases involving obviousness have dealt with the role of intermediates. In *In re Gyurik, 596 F.2d 1012, 201 U.S.P.Q. (BNA) 552 (CCPA 1979)*, the claimed thio compounds were rejected as *prima facie* obvious over a reference which generally disclosed such thio compounds as intermediates in the preparation of the corresponding sulfonyl compounds having the same general properties as those of the claimed compounds. The issue framed by the court was based solely upon the status of the claimed compounds as intermediates [**10] in the production of end products specifically named in the prior art.

In reversing the obviousness rejection the court said:

> [HN3] An element in determining obviousness of a new chemical compound is the motivation of one having ordinary skill in the art to make it. The motivation is not abstract, but practical, and is always related to the properties or uses one skilled in the art would expect the compound to have, if made. In re Stemniski . . . . The present obviousness rejection cannot stand without some basis in the expected properties of the claimed compounds.
>
> [HN4] In obviousness rejections based on close similarity in chemical structure, the necessary motivation to make a claimed compound, and thus the prima facie case of obviousness, rises from the expectation that compounds similar in structure will have similar properties. * * * No common-properties presumption rises from the mere occurrence of a claimed compound at an intermediate point in a conventional reaction yielding a specifically named prior art compound. That an intermediate/end-product relationship exists between a claimed compound and a prior art compound does not *alone* create a common-properties presumption. [**11] Absent that presumption or other evidence of motivation, it cannot be said that it would have been obvious to stop the process for synthesizing the disclosed end product and isolate the claimed intermediate. n14 [Citations omitted.]
>
> n14 The mere ability of a compound to act as an intermediate toward the production of other compounds does not alone constitute the sort of "property" that the cases on obviousness of chemical compounds contemplated.

Id. at 1018, 201 U.S.P.Q. at 557-8.

The court explained footnote 14 of *Gyurik* in *In re Magerlein, 602 F.2d 366, 373 n.15, 202 U.S.P.Q. 473, 479 n. 15 (CCPA 1979)*:

> Our recent statement . . . should not be read out of context as suggesting that the capacity to react to produce another compound is not, ipso facto, a property. The statement is merely a recognition that *there is no common-properties presumption or evidence of motivation to make the intermediate from the mere fact that an intermediate is in the chain of production of another compound.* [Emphasis added.]

Although *Gyurik* was not a case of obviousness based on structural similarity, and the facts of both *Gyurik* and *Magerlein* [**12] are different from those here, the dicta in those cases is helpful as a guide.

The PTO places great emphasis on the label "useful", contending that because the Oesterling final product is "useful", the intermediate sulfonyl chlorides are also "useful". That there is no common-properties presumption accorded to an intermediate and the end product of the reaction involving that intermediate necessarily means that there is no presumption that an intermediate's utility would be the same as that of the end product. Even if an unspecified "usefulness" or utility were all *Stemniski* requires, such utility could not be imputed from the fact that the Oesterling final product is "useful". The use of such labels, however, is meaningless because [HN5] we always look to "the subject matter [*707] as a whole" in determining whether the subject matter "would have been obvious at the time the invention was made." Further, a relevant property of a compound cannot be ignored in the determination of non-obviousness. *In re Papesch, 50 C.C.P.A. 1084, 315 F.2d 381, 391, 137 U.S.P.Q. 43, 51 (1963).*

Ultimately our analysis of the obviousness or nonobviousness of appellants' claimed compounds [**13] requires inquiry as to whether there is anything in the Oesterling reference which would suggest the expected properties of the claimed compounds or whether Oesterling discloses any utility for the intermediate sulfonyl chlorides which would support an expectation that the claimed compounds would have similar properties.

There is no disclosure that the Oesterling compounds would have any properties in common with those of appellants' compounds, as those properties of the former relate to the use of the compounds for base neutralization, catalysis, metal cleaning, and fuel. The mere fact that Oesterling's sulfonyl chlorides can be used as intermediates in the production of the corresponding sulfonic acids does not provide adequate motivation for one of ordinary skill in the art to stop the Oesterling synthesis and investigate the intermediate sulfonyl chlorides with an expectation of arriving at appellants' claimed sulfonyl halides for use as corrosion inhibiting agents, surface active agents, or leveling agents.

Oesterling does not teach the isolation and investigation of the intermediate sulfonyl chlorides, but rather discloses, as an optional step, the isolation and purification [**14] of the intermediate to obtain a purer sulfonic acid end product. The isolation and subsequent use of the intermediate sulfonyl chlorides in the production of the corresponding useful sulfonic acids is not motivation sufficient to support the structural obviousness rejection. The board has therefore failed to properly establish that the claimed compounds would have been prima facie obvious in view of Oesterling.

The decision of the board affirming the rejection of claims 13-22 is *reversed.*

REVERSED

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U.S. Appln. No. 10/770,639
Reference No. 25

Synergistic Effects of Interleukin-4 or Interleukin-13 and Tumor Necrosis Factor-α on Eosinophil Activation *In Vitro*

Werner Luttmann, Timm Matthiesen, Heinrich Matthys, and Johann Christian Virchow, Jr.

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Increased concentrations of tumor necrosis factor-α (TNF-α), interleukin (IL)-4, and IL-13 have been measured in bronchoalveolar lavage fluid (BALF) of patients with asthma following allergen provocation. In addition, these cytokines have also been reported to activate eosinophils *in vitro*. Although cytokine interactions have been postulated in the activation of eosinophils, the combined effects of cytokines on eosinophil activation remain poorly understood. Because activation of eosinophils has been regarded as a crucial event in the pathogenesis of asthmatic inflammation, we tested the hypothesis that IL-4 and IL-13 could enhance the effects of TNF-α on eosinophil activation. For this purpose, eosinophils from normal donors were purified and cultured in the presence of IL-4 or IL-13 and TNF-α. Eosinophil survival and surface expression of CD69 were assessed by flow cytometry. There was a concentration- and time-dependent upregulation in CD69 expression as well as eosinophil survival when eosinophils were incubated with IL-13, IL-4, or TNF-α. However, eosinophil viability and CD69 expression increased synergistically when eosinophils were incubated with IL-13 or IL-4 in the presence of TNF-α. This synergistic effect of IL-4 and IL-13 on CD69 expression was not limited to TNF-α but was also observed with IL-5. Our study provides evidence that IL-4 can activate eosinophils in a similar fashion as does IL-13. Furthermore, this study shows that the addition of IL-4 or IL-13 to TNF-α or IL-5 has synergistic effects on eosinophil activation, suggesting that the combined effects of different cytokines present in BALF following allergen provocation can enhance eosinophil activation *in vitro*. **Luttmann, W., T. Matthiesen, H. Matthys, and J. C. Virchow, Jr. 1999. Synergistic effects of interleukin-4 or interleukin-13 and tumor necrosis factor-α on eosinophil activation** ***in vitro*****.** Am. J. Respir. Cell Mol. Biol. 20:474–480.

Increased numbers of endobronchial eosinophils are a hallmark of bronchial asthma, and the concept that eosinophils play a crucial role in chronic asthmatic inflammation is generally accepted. Activation of eosinophils is therefore regarded as a crucial step in the initiation and maintenance of asthmatic inflammation as well as other diseases associated with atopy. Eosinophil numbers have been correlated with airflow obstruction (1) and bronchial hyperreactivity (2). The precise mechanisms regulating eosinophil activation *in vivo* remain unclear. Following allergen provocation in allergic asthma, an increase in eosinophils can be observed in the bronchoalveolar lavage fluid (BALF), where eosinophil numbers correlate with activated T lymphocytes and interleukin (IL)-5 as well as granulocyte–macrophage colony-stimulating factor (GM-CSF) concentrations (3). Accordingly, IL-5 and GM-CSF have been implicated in the regulation of eosinophil recruitment and activation (4–6). However, a number of other cytokines can be measured in increased concentrations following allergen provocation. Accordingly, mean concentrations of 1916 pg/ml of IL-4, 83 pg/ml of IL-13, and 449 pg/ml of tumor necrosis factor (TNF)-α have been measured in unconcentrated BALF 18 h after allergen challenge (7) and might therefore contribute to the regulation of eosinophil activation and survival *in vivo*. Recently, it has been shown that eosinophil chemotaxis can be stimulated by IL-4 (8), and eosinophil survival and activation can be enhanced by IL-13 (9). Both cytokines also selectively induce vascular cell adhesion molecule (VCAM)-1 expression on endothelial cells *in vitro* (10, 11) and may promote the antigen-induced, VCAM-1/very late antigen-4-dependent recruitment of eosinophils into tissue (12). TNF-α represents another cytokine that has been shown to influence eosinophil activation *in vitro* (13–16). TNF-α that can also be measured in increased concentrations in the lungs of pa-

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Abbreviations: bronchoalveolar lavage fluid, BALF; fetal calf serum, FCS; granulocyte–macrophage colony-stimulating factor, GM-CSF; immunoglobulin, Ig; interleukin, IL; phosphate-buffered saline, PBS; phycoerythrin, PE; specific mean fluorescence, SMF; recombinant human, rh; tumor necrosis factor-α, TNF-α; vascular cell adhesion molecule-1, VCAM-1.

Am. J. Respir. Cell Mol. Biol. Vol. 20, pp. 474–480, 1999
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tients with intrinsic bronchial asthma (17), as well as in allergic asthma following allergen provocation (3), is secreted by alveolar macrophages and mast cells, but also eosinophils (18–21), and has multifunctional properties including the induction of cell adhesion molecule expression, including that of intercellular adhesion molecule (ICAM)-1, endothelial cell leukocyte adhesion molecule-1, and VCAM on endothelial cells (22–24).

On the basis of the observation of the *in vivo* generation of multiple cytokines associated with activation of eosinophils *in vitro*, we compared the effects of IL-4, IL-13, and TNF-α on eosinophil activation *in vitro*. Furthermore, we tested the hypothesis that IL-4 or IL-13 in combination with TNF-α might augment eosinophil activation.

Materials and Methods

Reagents, Cytokines, and Antibodies

Percoll was obtained from Pharmacia (Uppsala, Sweden); phosphate-buffered saline (PBS) and RPMI from Seromed (Berlin, Germany); fetal calf serum (FCS) from Gibco (Paisley; UK); rhIL-4, rhIL-5, rhIL-13, and rhTNF-α from Bioconcept (Umkirch, Germany); L-glutamine, penicillin, streptomycin, ethylenediaminetetraacetic acid (EDTA), and propidium iodide were purchased from Sigma (Deisenhofen, Germany), phycoerythrin (PE)-conjugated anti-Leu 23 (CD69) from Becton-Dickinson (Heidelberg); PE-conjugated anti-immunoglobulin (Ig)G from Dako (Hamburg); CD16 microbeads and a magnetic cell separation system (MACS) from Miltenyi Biotec (Bergisch-Gladbach, Germany).

Purification of Eosinophils

Eosinophils were obtained from 100 ml EDTA-blood of healthy donors. Cells were separated according to a modified procedure initially described by Hansel and coworkers (25). Shortly after this, blood was diluted 1:1 with PBS and 22 ml aliquots were overlayered onto a 20 ml isotonic Percoll solution (density 1.080 g/ml) in 50 ml tubes and centrifuged for 30 min at 1,000 × *g* and 4°C. Differential cell counts were performed using Kimura stain. After centrifugation, the supernatant was removed and the mononuclear cells at the interface were aspirated. Erythrocytes and platelets were removed by hypotonic lysis (0.2% NaCl for 30 s). The granulocytes were washed twice in PBS containing 2% FCS. Eosinophils were separated by negative selection of neutrophils, using the MACS. The pellet was resuspended in 1 ml PBS/2% FCS, the number of granulocytes was counted, and 5 µl of CD16 microbeads per 1×10^7 neutrophils were added. Cells and microbeads were incubated for 30 min at 4°C with occasional mixing. The cell suspension was added onto the top of the separation column. Eosinophils were eluted with ice-cold PBS/2% FCS under magnetic influence. After separation, cells were washed twice in PBS/2% FCS. The purity of eosinophils was ≥ 97%, with some contaminating of mononuclear cells, as assessed by Kimura staining.

Cell Culture

Culture medium consisted of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 2% *N*-2-hydroxyethylpiperazine-*N*′-ethane sulfonic acid (Hepes). Eosinophils (1×10^6 cells/ml) were cultured at 37°C in a humidified atmosphere with 5% CO_2 either in culture medium alone or in the presence of cytokines. Before immunofluorescence labeling, the cells were washed twice in PBS/2% FCS.

Survival Assay

Survival of cultured eosinophils was assessed with propidium iodide. Cells (5×10^4) were washed once in PBS/2% FCS and resuspended in 100 µl of a propidium iodide solution (0.5 µg/ml dissolved in PBS). The relative proportion of viable to nonviable cells was determined by flow cytometry after analysis of at least 2,000 cells.

Flow Cytometric Analysis of CD69 Cell Surface Antigen

Expression of CD69 on eosinophils was measured as follows: 20 µl of cell suspension (5×10^4 cells) were incubated with 10 µl of PE-conjugated CD69 and PE-conjugated anti-IgG, respectively, for 30 min on ice. The cells were then washed once in PBS/2% FCS and resuspended in 100 µl of a propidium iodide solution (0.5 µg/ml in PBS). Flow cytometry was performed on at least 2,000 cells from each sample with a FACScan (Becton Dickinson, Heidelberg, Germany). To include only viable cells in the analysis, propidium iodide-positive, nonviable cells were excluded by appropriate gating in a separate fluorescence channel (FL3). Furthermore, PE-conjugated anti-CD69 antibodies were measured only on viable, propidium iodide negative cells (channel FL2). Nonspecific fluorescence was determined by incubating cells with mouse IgG of the same isotype but with irrelevant antigen specificity. The specific mean fluorescence (SMF) for each population was determined by subtracting the nonspecific fluorescence from the mean fluorescence measured with anti-CD69 antibody.

Statistical Analysis

Unless stated otherwise, the data in the text and figures are expressed as mean ± SEM. Comparisons between groups were performed using Wilcoxon's signed-rank test for paired samples. Statistical significance was assumed at $P < 0.05$.

Results

Induction of CD69 Expression on Eosinophils by IL-13 and IL-4

When purified eosinophils were labeled with anti-CD69 antibody, expression of CD69 surface antigen was generally not detectable. However, there was a low-level induction of CD69 expression when isolated blood eosinophils (5×10^4 cells/ml) were cultured for 6 h (2.35 ± 0.81 SMF) in medium (Figure 1). Incubation of purified eosinophils with increasing concentrations of IL-4 or IL-13 (between 1 and 100 ng/ml) for 6 h caused a concentration-dependent increase in CD69 surface expression (Figure 1). In contrast to the effects of IL-4 on CD69 expression, which reached a maximum at 10 ng/ml, there was a linear increase in CD69 expression following incubation of eosinophils

with IL-13 over the dose range investigated. CD69 surface expression increased after incubation of eosinophils with 1, 10, and 100 ng/ml IL-13 to 5.1 ± 1.5, 8.03 ± 1.81, and 9.82 ± 2.03 SMF, respectively. In comparison, IL-4 used in the same concentrations increased the CD69 surface expression to 8.18 ± 2.01, 8.93 ± 1.69, and 8.92 ± 1.98 SMF, respectively (Figure 1). The observed differences in CD69 expression following incubation with IL-4 and IL-13 were consistently significantly different when compared with unstimulated control cells (each $P < 0.01$).

The time-dependent kinetics of cytokine-induced CD69 expression on eosinophils were studied in a separate set of experiments. For this purpose, eosinophils were incubated in the presence of 10 ng/ml IL-13 or IL-4 for 6 and 24 h, after which CD69 expression was analyzed. Following incubation with IL-13, the SMF for the CD69 expression increased to 6.8 ± 1.29 after 6 h and 4.18 ± 1.06 SMF after 24 h, which was significantly different from control cells ($P < 0.01$). Similar results were obtained following incubation with IL-4; SMF for CD69 reached 6.87 ± 1.24 after 6 h and 3.99 ± 0.81 after 24 h ($P < 0.001$ compared with control cells). In contrast, there was only a minor change in the CD69 expression on control eosinophils incubated in medium alone (1.23 ± 0.55 SMF after 6 h and 1.46 ± 0.58 SMF after 24 h) (Figure 2A).

Coincubation of TNF-α with IL-4 and IL-13

Compared with IL-13 or IL-4, the effects of 10 ng/ml TNF-α on CD69 expression were less pronounced (3.57 ± 0.82 SMF after 6 h and 3.61 ± 1.07 SMF after 24 h of incubation), but were still significantly elevated compared with unstimulated control cells ($P < 0.01$) (Figure 2A). However, when eosinophils were incubated in the presence of either IL-4 or IL-13 with TNF-α, a synergistic upregulation of CD69 expression was observed. As shown in Figure 2A, CD69 surface expression on eosinophils following incubation with 10 ng/ml of TNF-α and 10 ng/ml IL-13 increased to 13.21 ± 2.11 SMF after 6 h of incubation and remained at 9.68 ± 1.99 SMF after 24 h. Almost identical results for CD69 expression were obtained when IL-4 (10 ng/ml) was used (13.84 ± 2.21 SMF after 6 h incubation and 10.83 ± 2.37 SMF after 24 h). The observed differences in CD69 expression following coincubation of eo-

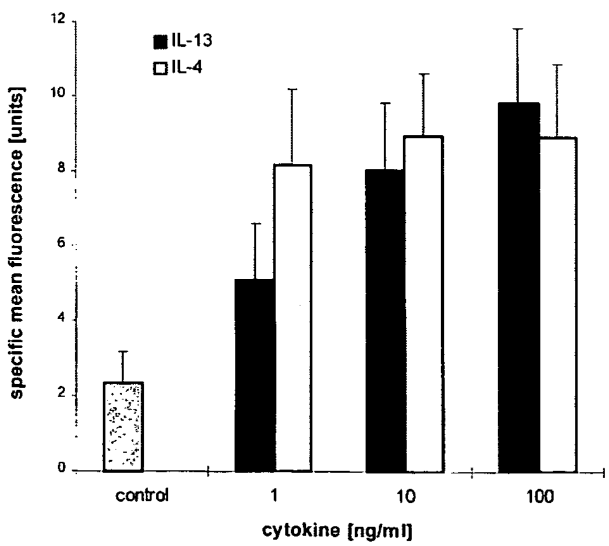

Figure 1. Dose-response analysis of effect of IL-13 and IL-4 on CD69 expression on eosinophils. Isolated peripheral blood eosinophils were stimulated with 1 to 100 ng/ml of either IL-13 or IL-4. CD69 expression was measured after 6 h in culture. Data are given as SMF ± SEM obtained from nine independent experiments ($P < 0.01$ compared with control).

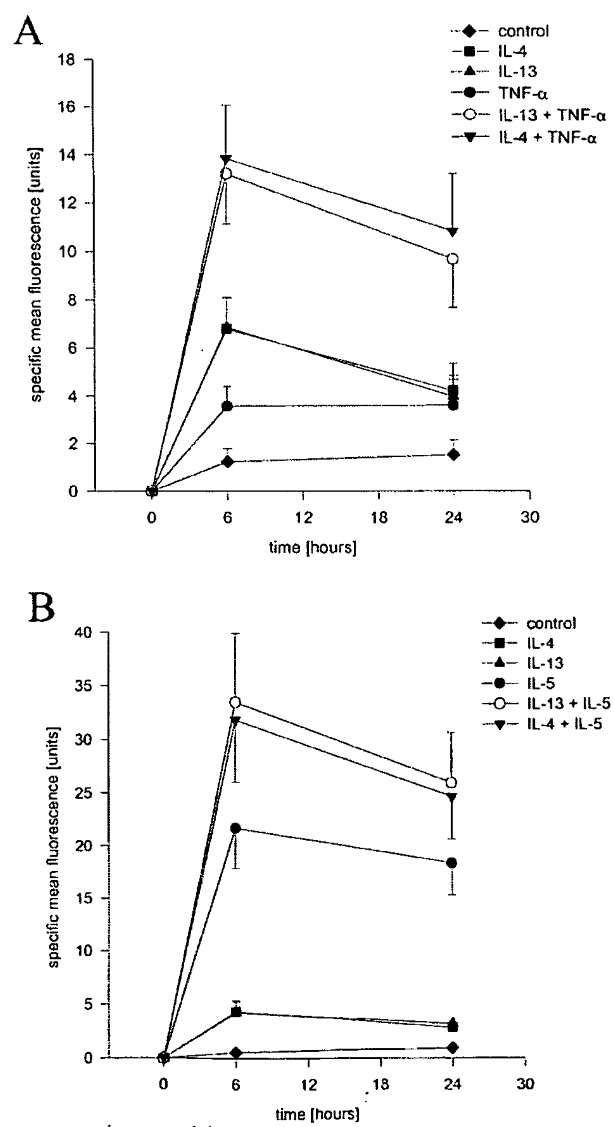

Figure 2. Time-dependent expression of CD69 on eosinophils following incubation with IL-4 or IL-13 and TNF-α, or IL-5 alone or with combinations of IL-4 or IL-13 and TNF-α (*A*) or IL-5 (*B*) for 6 and 24 h, respectively. Cells cultured in medium alone served as controls. Data are given as SMF ± SEM obtained from 15 (*A*) and 11 (*B*) independent experiments (each $P < 0.01$).

sinophils with IL-4 or IL-13 in combination with TNF-α consistently reached statistical significance when compared with CD69 expression measured after incubation with IL-4, IL-13 or TNF-α alone (each $P < 0.01$).

In a separate set of experiments, the observed synergistic effects of IL-4 or IL-13 with TNF-α on CD69 expression were compared with those of IL-5, a potent activator of eosinophils. As shown in Figure 2b, incubation of eosinophils with IL-5 alone increased CD69 expression significantly over that of control cells ($P < 0.01$, $n = 11$). However, there was a further, statistically significant increase in CD69 expression when IL-4 or IL-13 was added to IL-5 (Figure 2b, $P < 0.01$). Figure 3 displays representative histograms of the IL-4- and IL-13-induced CD69 expression with or without TNF-α or IL-5.

The synergistic effects of IL-4 or IL-13 and TNF-α on CD69 expression were also demonstrated in a concentration-dependent fashion when increasing concentrations of TNF-α ranging from 0.1 to 100 ng/ml were added to either IL-4 or IL-13 (each 10 ng/ml) (Figure 4). CD69 expression on cultured eosinophils increased from 163 ± 113 SMF for cells incubated in medium alone to 586 ± 173 SMF when cells were cultured in the presence of 100 ng/ml TNF-α ($P < 0.05$). In contrast, CD69 expression was markedly upregulated when cells were incubated with IL-4 or IL-13 (each 10 ng/ml) (1032 ± 184 SMF for IL-4 and 958 ± 216 SMF for IL-13). This increase in CD69 expression was dose-dependently enhanced to 1888 ± 488 SMF for IL-4 and 1973 ± 401 SMF for IL-13 when TNF-α (100 ng/ml) was added. This was statistically significant when compared with cells incubated with IL-4 or IL-13 alone ($P < 0.05$, except for 0.1 ng/ml TNF-α, where $P = 0.19$) (Figure 4).

Influence of TNF-α on IL-13- and IL-4-enhanced Eosinophil Survival

To substantiate further the activating effects of IL-4 or IL-13 and TNF-α on cultured eosinophils, cell viability was

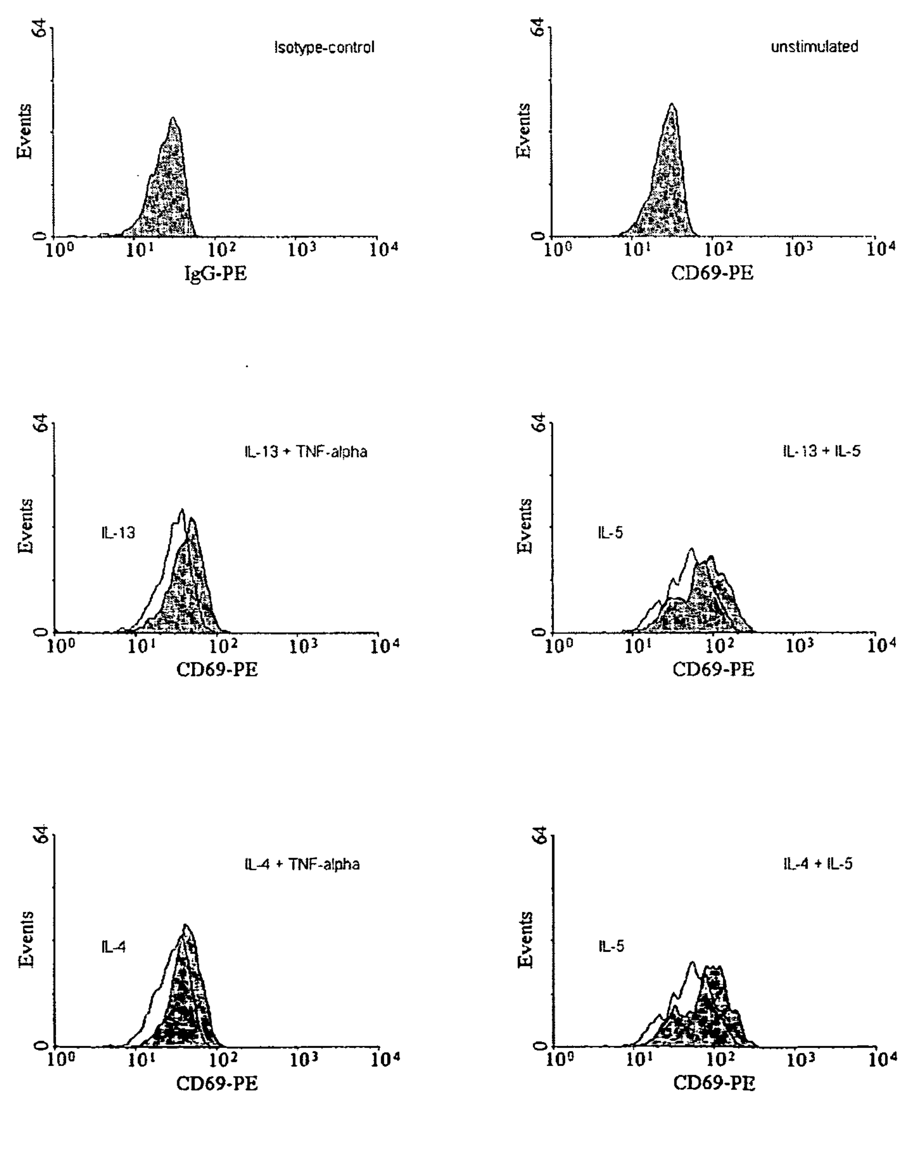

Figure 3 Enhancement of CD69 expression following stimulation with cytokines. Isolated peripheral blood eosinophils were incubated for 6 h with either IL-4, IL-13, TNF-α, or IL-5 alone, or stimulated with IL-4 or IL-13 and TNF-α or IL-5 (each 10 ng/ml). CD69 expression was measured by flow cytometry. The figure shows representative histograms of at least 11 independent experiments.

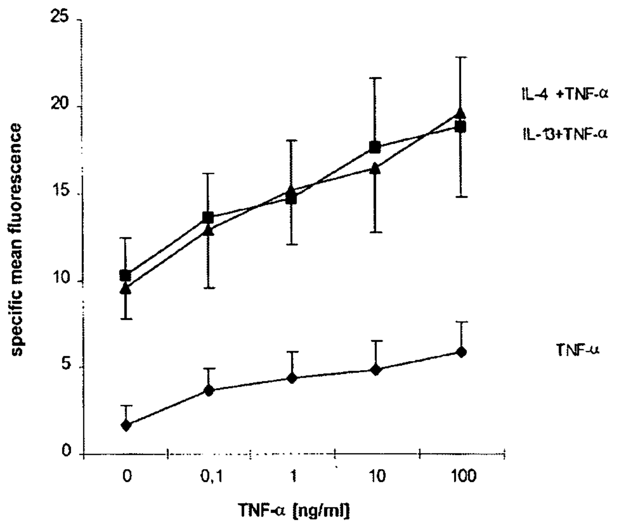

Figure 4. Dose-response curve for CD69 expression on human eosinophils following incubation with TNF-α alone or in combination with IL-4 or IL-13. Isolated human peripheral blood eosinophils were incubated in the presence of increasing concentrations of TNF-α (1 to 100 ng/ml) for 6 h with or without IL-4 or IL-13 (10 ng/ml). After being labeled with anti-CD69, eosinophils were analyzed by flow cytometry. Data are given as SMF ± SEM obtained from seven independent experiments ($P < 0.05$).

measured following incubation of eosinophils (5×10^6/ml) in RPMI over 4 d in the absence or presence of IL-13 or IL-4 (10 ng/ml each) alone or in combination with TNF-α (10 ng/ml). Eosinophil viability was assessed by propidium

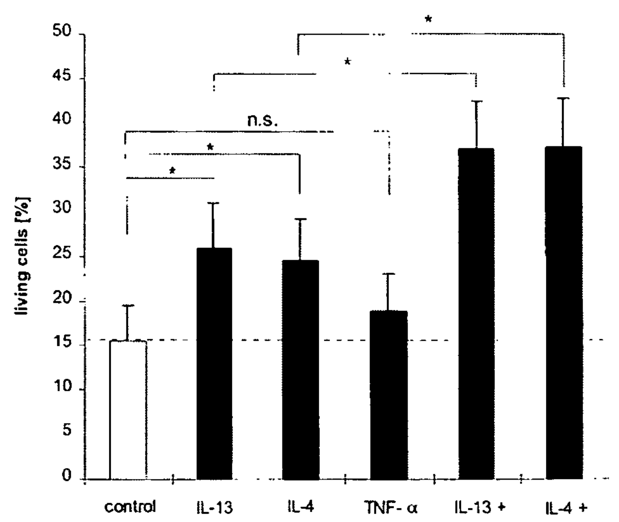

Figure 5. Increase in eosinophil survival *in vitro* following incubation with IL-13 or IL-4 and TNF-α. Eosinophils were incubated for 4 d in the presence of 10 ng/ml of either IL-13, IL-4, or TNF-α alone or with combinations of IL-13 or IL-4 and TNF-α. Cell viability was analyzed by flow cytometry using propidium iodide exclusion. Data are shown as percent viable cells ± SEM from 16 individual experiments (n.s. = nonsignificant, * = $P < 0.001$).

iodide exclusion measured by flow cytometry. After 4 d in culture, there were only 15.5 ± 4.0% viable eosinophils present in culture. In contrast, when eosinophils were cultured in the presence of IL-13 or IL-4 (10 ng/ml each), survival after day 4 was increased to 26.0 ± 5.0% and 24.5 ± 4.6% (each $P < 0.001$), respectively. In comparison, eosinophil survival following incubation with TNF-α alone was 18.9 ± 4.1%, which was not statistically significantly different from cells incubated in medium alone. However, costimulation of eosinophils with IL-13 or IL-4 in the presence of TNF-α (10 ng/ml each) increased survival of eosinophils after 4 d in culture to 37.1 ± 5.3% and 37.3 ± 5.5% (each $P < 0.001$), respectively (Figure 5). Thus, the mean change in eosinophil survival expressed as percentage of unstimulated control cells was 21.9% for TNF-α, 67.7% for IL-13, and 58.1% for IL-4, whereas the coincubation of IL-13 or IL-4 in the presence of TNF-α increased the mean number of viable cells corrected for the appropriate control by 139.4% and 140.6%, respectively.

Discussion

In this study, we investigated the effects of IL-13, IL-4, and TNF-α on eosinophil survival and CD69 expression. Because eosinophil viability *in vitro* decreases rapidly in the absence of eosinophil-activating cytokines, the observed increase in eosinophil viability following incubation with cytokines added to cell cultures is suggestive of eosinophil-activating properties. Similarly, induction of CD69 expression on eosinophils has been reported as an indication of eosinophil activation (26). Therefore, our results, which show an increase in eosinophil viability as well as CD69 expression on cultured eosinophils, enlarge on previous observations in which we have reported that IL-13 can increase eosinophil survival and CD69 expression *in vitro* (9) by demonstrating similar results for IL-4. Therefore, to the best of our knowledge, this is the first report showing that IL-4 can directly activate normal eosinophils and our findings challenge a recent report in which eosinophil survival was enhanced following incubation with IL-13 but not with IL-4 (27). The reasons for these differences remain unclear. In a different study, an increase in eosinophil chemotaxis has been reported for eosinophils from patients with atopic dermatitis following incubation with IL-4, which, however, was not observed with eosinophils from normal donors (8). With respect to eosinophil viability and CD69 expression as markers for eosinophil activation, we were not able to substantiate these differential findings for IL-4 and IL-13. Thus, in our hands, IL-4 as well as IL-13, both of which have been implicated in the regulation of IgE synthesis (28–30), were able to induce eosinophil activation and could thus influence the humoral as well as the cellular arm of allergic hypersensitivity reactions.

TNF-α, another cytokine present in elevated concentrations following allergen provocation in allergic asthma, has been shown to increase expression of ICAM-1 on cultured eosinophils from normal donors (13, 14). Furthermore, incubation of eosinophils from normal donors induced CD4 (16) and the release of reactive oxygen species (15). Here, we report that incubation of eosinophils with TNF-α has a weak effect on eosinophil survival as well as

CD69 expression. These effects of TNF-α on eosinophils were consistently weaker than those of IL-4 and IL-13.

Interestingly, when eosinophils were incubated with a combination of either IL-4 or IL-13 and TNF-α, there was a marked, synergistic upregulation of eosinophil activation as measured by CD69 expression. This increase in eosinophil activation compared favorably with the effects of IL-5, and we were able to show an additional effect of IL-4 or IL-13 to that of IL-5. Although the increase in CD69 expression by eosinophils following incubation with IL-5 was significantly higher than that observed following stimulation with IL-4 or IL-13 and TNF-α, our results demonstrate that IL-4 and IL-13 can further enhance CD69 expression even in the presence of a potent activator of eosinophil function such as IL-5. In addition, eosinophil survival was enhanced when IL-4 or IL-13 was incubated with TNF-α, supporting the hypothesis that TNF-α can enhance eosinophil activation by IL-4 and IL-13. Similar effects have not been reported previously for eosinophils.

A synergistic effect of IL-13 and TNF-α has recently been shown for the induction of VCAM-1 on endothelial cells *in vitro* (31, 32). Similar results have been obtained for IL-4 (33). Because VCAM-1 appears to be crucial for the selective recruitment of eosinophils and lymphocytes to the site of inflammation in allergic diseases (12), and can be upregulated synergistically by IL-4 or IL-13 and TNF-α, our results provide evidence that the combination of IL-4 or IL-13 with TNF-α also has direct activating effects on eosinophils.

IL-13, as well as IL-4 and TNF-α, can be found in elevated concentrations following allergen provocation in allergic asthma (3, 34-37). It has been proposed that these cytokines are produced locally in the bronchoalveolar compartment by several cell types following allergen provocation (34, 36-38). Therefore, our results suggest that different cytokines present in elevated concentrations in BALF following allergen provocation, especially IL-4, IL-13, and TNF-α, can contribute synergistically to activate eosinophils. Even though these effects might not reach the magnitude of the classic eosinophilopoietins IL-3, IL-5, and GM-CSF, our results provide evidence that the combination of other cytokines might also contribute to the activation of eosinophils in allergic asthma.

In this study, we have shown that there is no difference in the effects of IL-13 and IL-4 on human eosinophils from normal donors. Because IL-13 and IL-4 share common receptor subunits, it can be speculated that the effects are mediated by similar receptor-dependent pathways. In contrast to our findings, differential effects for IL-4 and IL-13 have been reported for IL-1Ra production by neutrophils (39). Although in this study IL-4 synergized with TNF-α to induce IL-1Ra production by human neutrophils, IL-13 did not modulate TNF-α–induced IL-1Ra production (39). Thus, although the effects of IL-4 or IL-13 in combination with TNF-α are almost identical with respect to eosinophil activation and endothelial cell adhesion molecule expression, differential effects of these cytokines might be operant on other cell populations.

In conclusion, we provide evidence that IL-4 and IL-13 have similar effects on eosinophil activation, and that these effects can be synergistically enhanced by TNF-α. Because these cytokines have been measured in increased concentrations following allergen provocation in asthma, it can be speculated that these cytokines could contribute to the orchestration of eosinophil inflammation in allergic asthma.

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Reversing Tumor Immune Suppression with Intratumoral IL-12: Activation of Tumor-Associated T Effector/Memory Cells, Induction of T Suppressor Apoptosis, and Infiltration of $CD8^+$ T Effectors[1]

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A single intratumoral injection of IL-12 and GM-CSF-loaded slow-release microspheres induces T cell-dependent eradication of established primary and metastatic tumors in a murine lung tumor model. To determine how the delivery of cytokines directly to the microenvironment of a tumor nodule induces local and systemic antitumor T cell activity, we characterized therapy-induced phenotypic and functional changes in tumor-infiltrating T cell populations. Analysis of pretherapy tumors demonstrated that advanced primary tumors were infiltrated by $CD4^+$ and $CD8^+$ T cells with an effector/memory phenotype and $CD4^+CD25^+Foxp3^+$ T suppressor cells. Tumor-associated effector memory $CD8^+$ T cells displayed impaired cytotoxic function, whereas $CD4^+CD25^+Foxp3^+$ cells effectively inhibited T cell proliferation demonstrating functional integrity. IL-12/GM-CSF treatment promoted a rapid up-regulation of CD43 and CD69 on $CD8^+$ effector/memory T cells, augmented their ability to produce IFN-γ, and restored granzyme B expression. Importantly, treatment also induced a concomitant and progressive loss of T suppressors from the tumor. Further analysis established that activation of pre-existing effector memory T cells was short-lived and that both the effector/memory and the suppressor T cells became apoptotic within 4 days of treatment. Apoptotic death of pre-existing effector/memory and suppressor T cells was followed by infiltration of the tumor with activated, nonapoptotic $CD8^+$ effector T lymphocytes on day 7 posttherapy. Both $CD8^+$ T cell activation and T suppressor cell purge were mediated primarily by IL-12 and required IFN-γ. This study provides important insight into how local IL-12 therapy alters the immunosuppressive tumor milieu to one that is immunologically active, ultimately resulting in tumor regression. ***The Journal of Immunology,*** **2006, 177: 6962–6973.**

It is now well-established that tumor vaccines can successfully promote tumor-specific T cell responses in both preclinical and clinical studies (1, 2). However, induction of antitumor T cell immunity rarely results in effective eradication of established disease in murine models or patients (3). In the majority of studies, posttherapy antitumor activity is assessed by monitoring of peripheral T cell immunity (4). Whereas this strategy provides a convenient and accurate method for quantification of tumor-specific T cells, it does not predict whether these cells will maintain effector activity once they encounter the highly immune-suppressive tumor milieu (5, 6). The mechanisms that mediate immune suppression within the tumor microenvironment are complex (6). Tumors actively produce immune inhibitory cytokines, enzymes, and death receptor ligands, and are enriched in T suppressor cells (6). It is thus likely that perturbation of the equilibrium that exists between immune-suppressive factors and antitumor lymphocytes within the tumor microenvironment is critical to therapeutic success. For example, elimination of T suppressor cells from tumors uncovers natural antitumor responses and results in tumor regression (7–9). Therefore, strategies that combine tumor vaccination with modulation of the suppressive factors within the tumor microenvironment represent a potentially effective approach in enhancing the success of therapeutic vaccination in cancer patients.

The cytokine milieu within the tumor microenvironment is critical to the balance between tumor-mediated immune suppression and the antitumor activity of infiltrating leukocytes (10). For example, advanced tumors, which are rich in immune-suppressive cytokines such as TGFβ and IL-10, not only suppress the antitumor activity of infiltrating leukocytes but can subvert their function to their advantage (11). Local and sustained delivery of proinflammatory cytokines into tumors, in contrast, can reverse this balance in favor of antitumor immunity (10). In this context, induction of acute inflammatory activity within the tumor not only promotes local tumor regression by activating tumor-associated lymphocytes, but can also prime systemic responses via the release of tumor Ags to the draining lymph nodes (LN)[3] in the presence of inflammatory "danger" signals (12). To this end, previous studies in our laboratory demonstrated that local and sustained delivery of IL-12 to the microenvironment of a progressively growing tumor from controlled-release microsphere adjuvants (in situ vaccination) promoted the complete eradication of established tumors and the development of long-term antitumor T cell immunity (13).

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[3] Abbreviations used in this paper: LN, lymph node; TIL, tumor-infiltrating lymphocyte; FNA, fine needle aspirate; Ct, threshold cycle; TDLN, tumor-draining LN; AICD, activation-induced cell death; FasL, Fas ligand; wt, wild type; GKO, IFN-γ-knockout.

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Subsequently, we demonstrated that a single intratumoral injection of IL-12 and GM-CSF microspheres was superior to either cytokine alone in inducing the development of systemic antitumor T cell immunity and the eradication of disseminated disease in a murine spontaneous lung metastasis model (14). The antitumor activity was found to be mediated by T and NK cells in this model.

Although earlier studies established the antitumor efficacy of IL-12/GM-CSF microsphere therapy, the cellular and molecular basis of the posttreatment immune activity within the tumor microenvironment was not elucidated. The ability of IL-12 to stimulate both innate (NK, NKT) and adaptive (T cell) immunity (15), and that of GM-CSF to augment Ag presentation are well-established (16). Numerous preclinical and clinical studies have demonstrated that IL-12 mediates tumor regression by promoting Th1 responses, by increasing $CD8^+$ T cell, NKT cell, NK cell, and granulocyte cytotoxicity, and by inhibiting angiogenesis (15, 17). GM-CSF, in contrast, augments the generation and recruitment of dendritic cells, macrophages, granulocytes, and NKT cells (10, 16). In the majority of these studies, monitoring of cytokine-induced immune activity within the tumor microenvironment was limited to histological analyses, providing snapshots of leukocytic infiltrates. A quantitative characterization of the lymphocyte activation kinetics as induced by intratumoral cytokine administration, particularly with specific reference to tumor immune suppression, has not been conducted. To this end, we characterized the long-term kinetics and the phenotypic/functional properties of tumor-associated T cells to determine how intratumoral delivery of IL-12/GM-CSF induces antitumor immunity. The results demonstrate that local release of IL-12/GM-CSF reverses tumor immune suppression via the modulation of a complex multicomponent T cell network, including the activation of pre-existing tumor-associated effector/memory T cells, elimination of T suppressor cells, and the priming of a secondary $CD8^+$ T effector response.

Materials and Methods

Mice and tumor cells

Line-1, a BALB/c lung alveolar carcinoma cell line, was maintained in DMEM/F-12 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (Equitech-bio), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, and 0.1 mM sodium pyruvate (Mediatech). Female BALB/c mice at 6–8 wk of age were obtained from Taconic Farms. IFN-γ-knockout BALB/c mice were purchased from The Jackson Laboratory. All studies were approved by the Institutional Animal Care and Use Committee of the University of Louisville.

Cytokines and microspheres

Recombinant murine IL-12 (2.7×10^6 U/mg) was a gift from Wyeth. Recombinant murine GM-CSF (5×10^6 U/mg) was purchased from PeproTech. Preparation of cytokine-encapsulated biodegradable polymer microspheres has been described in detail previously (14).

Preparation of single-cell suspensions from tumors

Tumors were induced by s.c. injection of 1×10^6 viable tumor cells in 0.1 ml of sterile PBS behind the neck just above the scapula (14). Thirteen to 15 days later, when tumors reached 400–500 mm^3 in size, mice were treated with cytokine-encapsulated microspheres as described previously (14). Experimental groups received IL-12/GM-CSF-encapsulated microspheres (1.5 μg of each cytokine in 12 mg of particles suspended in 50 μl PBS), whereas control mice received blank microspheres. At selected timepoints after microsphere treatment, tumors were removed from mice and single-cell suspensions were prepared by enzymatic digestion. Resected tumors were weighed, minced into small (1–2 mm^3) pieces with a scalpel, and immersed in 10 ml of digestion mixture (5% FBS in RPMI 1640, 0.5 mg/ml collagenase A (Roche Diagnostic), 0.2 mg/ml hyaluronidase, type V (Sigma-Aldrich), and 0.02 mg/ml DNase I (Sigma-Aldrich)) per 0.25 g of tumor tissue. This mixture was incubated at 37°C for 45 min on a rotating platform. The resulting cell suspensions were filtered sequentially through 70- and 40-μm cell strainers (BD Falcon) and washed with 5% FBS in RPMI 1640. RBC were lysed by brief incubation in 0.15 M ammonium chloride solution, and cell debris/dead cells were removed by centrifugation on Lympholyte-M gradients as recommended by the manufacturer (Cedarlane).

Flow cytometry

Single-cell suspensions obtained from samples were labeled with Abs to various T cell markers using standard staining methods (18) and were analyzed on a four-color FACSCalibur flow cytometer (BD Biosciences). The following panel of commercially available and directly fluorochrome-conjugated anti-mouse mAbs was included in this study: CD3 (clone 17A2), CD4 (clone GK1.5, RM4-5), CD8 (clone 53-6.7), CD25 (clone PC61), CD69 (clone H1.2F3), CD43 (clone 1B11), CD44 (clone IM7), CD49b/pan-NK (clone DX5α), CD62L (clone MEL-14), CD95 (clone Jo2), CD95 ligand (clone MFL3), and TCR β-chain (clone H57-597). To evaluate membrane TGFβ expression, the staining was performed with biotinylated anti-TGFβ1 mAb (clone A75-3) followed by streptavidin-PE labeling. All Abs and mouse Fc block (clone 2.4G2) were purchased from BD Pharmingen. Flow cytometry data were analyzed using CellQuest software (BD Biosciences).

Intracellular cytokine staining

Single-cell suspensions of primary tumors were prepared as described above. For intracellular staining of IFN-γ or IL-10, tumor-infiltrating lymphocytes (TIL) were stimulated with PMA (5 ng/ml) and ionomycin (0.5 μg/ml) for 5 h. Two hours before harvesting, 0.5 μl of BD Golgistop (BD Pharmingen) was added to every 1 ml of cell culture (1×10^6 live cells/ml). After two washes, intracellular IL-10 and IFN-γ staining was performed according to the manufacturer's instructions using the BD Cytofix/Cytoperm Plus kit and PE-conjugated anti-mouse IL-10 or IFN-γ (BD Pharmingen). For granzyme B, cells were directly pretreated with FcR block and stained with Abs targeting cell surface markers (CD4 and CD25 or CD8). Granzyme B staining was performed using eBioscience Fixation and Permeabilization kit and PE anti-mouse granzyme B (eBioscience). For detection of apoptosis, cells were first stained for the expression of the respective surface markers and then with anti-Annexin V-allophycocyanin Ab according to the manufacturer's protocol (Annexin V apoptosis detection kit; BD Pharmingen). For detection of anti- and proapoptotic proteins, PE-labeled anti-Bcl-x_L (clone H-5) was purchased from Santa Cruz Biotechnology. Active caspase-3 (C92-605) and Bcl-2 (3F11) detection was performed using the BD Pharmingen kits according to the manufacturer's protocol. Foxp3 expression was analyzed using an anti-mouse Foxp3-PE staining kit according to the manufacturer's protocol (eBioscience).

In vitro T suppressor assay

Total $CD4^+$ TIL were purified from single-cell suspensions using magnetic cell sorting. Cells were incubated with anti-CD4 microbeads (Miltenyi Biotec) and passed through the autoMACS separator according to the manufacturer's instructions. Tumor-infiltrating $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated from the enriched population by FACS sorting. Responder cells were obtained from LN and spleens of naive BALB/c mice, and prepared as described previously (19). $CD4^+$ T cells were enriched on a CD4 cell-enrichment column (R&D Systems), then labeled with PE-anti-CD25 Ab and incubated with anti-PE beads (Miltenyi Biotec). $CD4^+CD25^+$ T cell purity was consistently >90%. Subsequently, $CD4^+CD25^-$ responder T cells (1×10^4/well in 96-well round-bottom plates) were cultured for 3 days at 37°C in 5% CO2 in the presence of irradiated spleen cells as APC (1×10^5/well), anti-CD3 Ab at 0.5 μg/ml, with or without $CD4^+CD25^+$ T cells at a ratio of four $CD4^+CD25^+$ T cells to one responder cell. The cell cultures were pulsed on day 3 with 0.5 μCi of [^{3}H]thymidine for the last 18 h.

Quantitative real time-PCR

Fine-needle aspirates (FNA) were obtained by aspirating four quadrants of each tumor with a 23¾-gauge needle attached to a 1.0-ml syringe. Tissue samples were discharged into TRIzol reagent (Invitrogen Life Technologies), total RNA was isolated and was reverse-transcribed with TaqMan Reverse Transcription Reagents (Applied Biosystems). IFN-γ, CD4, CD8, and GAPDH mRNA levels were quantified by real-time RT-PCR amplification using the Mx3000PTM Real-Time PCR System (Stratagene) as recommended by the manufacturer. Briefly, cDNA was amplified in a 25-μl reaction mixture containing 12.5 μl of SYBR Green PCR Master Mix (Applied Biosystems), 100 ng of cDNA template, and selected primers (200 nM) using the recommended cycling conditions (denaturation at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). The primer sequences, designed with Primer Express software (Applied Biosystems), were as follows:

IFN-γ, 5′-GGCACAGTCATTGAAAGC-3′ (forward) and 5′-TGCCAGTTCCTCCAGATA-3′ (reverse); CD8, 5′-GCTACCACAGGAGCCGAAAG-3′ (forward) and 5′-TGGGCTTGCCTTCCTGTCT-3′ (reverse); CD4, 5′-GGTGGAGTTGTGGGTGTTCAA-3′ (forward) and 5′-CAGGCTCTGCCCTTGCAA-3′ (reverse); GAPDH, 5′-TCCTTGATTTCTGGGCCATG-3′ (forward) and 5′-TCTTCTGGGTGGCAGTGATG-3′ (reverse). Relative quantification of mRNA expression was calculated by the comparative threshold cycle (*Ct*) method (20). The relative target quantity, normalized to an endogenous control (GAPDH) and relative to the day zero calibrator, is expressed as $2^{\Delta\Delta Ct}$ (fold), where $\Delta Ct = Ct$ of the target gene − Ct of endogenous control gene, and $\Delta\Delta Ct = \Delta Ct$ of samples for target gene − ΔCt of the zero day calibrator for the target gene.

Statistical analysis

All data were analyzed using unpaired Student's *t* test analysis. A *p* value of 0.05 or less was considered significant.

Results

Advanced primary tumors are infiltrated by $CD8^+$ and $CD4^+CD25^-$ effector/memory T cells and $CD4^+CD25^+$ suppressor T cells

In initial studies, TIL populations from untreated tumors were analyzed to determine the extent of pre-existing T cell infiltration. Tumors were induced and allowed to grow to ~400–500 mm^3 in size (13–15 days), representing a well-established tumor. Flow cytometric analysis of single-cell suspensions prepared from these tumors revealed a substantial (51%) T cell (TCR^+CD3^+) component within the TIL, with an overall CD4:CD8 ratio of 5:1 (data not shown). All intratumoral $CD8^+$ T cells expressed high levels of CD44, demonstrating Ag experience (Fig. 1*A*). To determine whether these cells displayed an effector or memory phenotype, CD43 expression was evaluated. CD43 is up-regulated rapidly on effector T cells upon activation (similar to CD44); however, its expression declines (unlike CD44) as effector T cells develop into memory cells (21). The great majority of the $CD8^+$ T cells were positive for CD43, consistent with an effector phenotype (Fig. 1*A*). In contrast, early activation markers CD25 and CD69, which are expressed transiently by fully activated effector T cells (22), were found to be expressed at low levels, indicating late- or postpeak effector status. The presence of low levels of CD127, which is expressed by either naive or central memory but not by effector cells (22), also suggested a late effector phenotype. Finally, low CD62L expression, a marker that is up-regulated on either naive or central memory T cells, was again consistent with a T effector/memory phenotype.

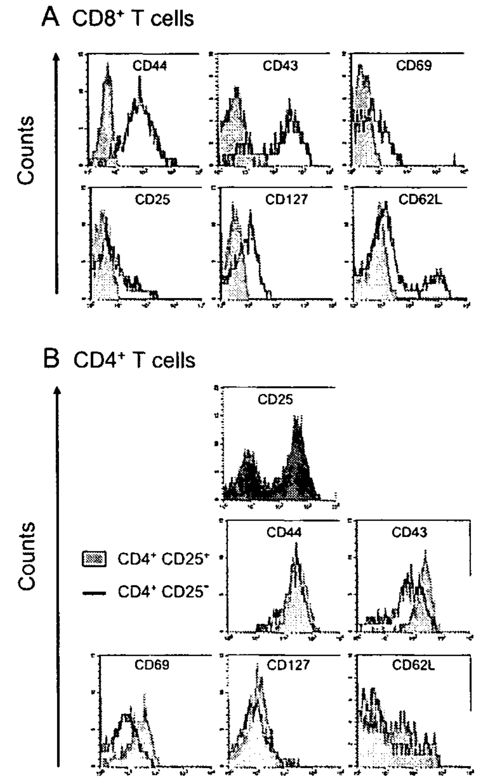

FIGURE 1. Analysis of membrane phenotype markers on tumor-infiltrating $CD8^+$ and $CD4^+$ T cells. *A*, $CD8^+$ T cells. Single-cell suspensions prepared from tumors were analyzed after gating on the mononuclear lymphocyte population. $CD8^+$ cells were gated on and analyzed for the expression of CD25, CD43, CD44, CD62L, CD69, and CD127 (solid lines). Filled histograms represent isotype controls. *B*, $CD4^+$ T cells. *Top panel*, $CD25^+$ and $CD25^-$ subpopulations after gating on $CD4^+$ cells. *Lower panels*, $CD4^+CD25^+$ or $CD4^+CD25^-$ cells were gated on separately and analyzed for the expression of CD43, CD44, CD62L, CD69, and CD127. Filled histograms, $CD4^+CD25^+$ cells; solid lines, $CD4^+CD25^-$ cells.

Analysis of tumor-associated $CD4^+$ T cells demonstrated that tumors were infiltrated with two distinct populations, i.e., $CD25^-$ and $CD25^+$ subsets, corresponding to 40 and 60% of the $CD4^+$ T cells, respectively (Fig. 1*B*, *top panel*). The $CD4^+CD25^-$ and $CD4^+CD25^+$ populations were analyzed separately with regard to their CD44, CD43, CD69, CD127, and CD62L expression. This approach established that the $CD4^+CD25^-$ T cells had a phenotype that was similar to that of the $CD8^+$ T effector/memory cells (Fig. 1*B*). $CD4^+CD25^+$ T cells, in contrast, expressed significantly higher levels of CD43 and CD69, consistent with an activated effector cell phenotype. CD62L expression was mixed, suggesting that these cells were likely circulating between the tumor and the tumor-draining LN (TDLN).

Next, we undertook functional analysis of tumor-associated $CD8^+$ and $CD4^+$ T cell populations. $CD8^+$ T cells were analyzed with respect to their ability to secrete IFN-γ and granzyme B to determine whether they were active and displayed cytotoxic function. Stimulation with PMA and ionomycin resulted in IFN-γ production by more than half of the $CD8^+$ T cells, suggesting that a significant portion of the $CD8^+$ T cells were functional with regard to cytokine secretion (Fig. 2*A*). In contrast, these cells did not produce granzyme B, demonstrating that they were impaired in cytotoxic function.

Similar to the $CD8^+$ T cells, in vitro stimulation of $CD4^+$ T cells resulted in the production of IFN-γ (but not IL-10) by a significant number of $CD4^+CD25^-$ T cells consistent with a type 1 Th (Th1) cell phenotype (Fig. 2*B*). $CD4^+CD25^+$ T cells did not produce IFN-γ upon stimulation, suggesting that they represented either activated type 2 Th (Th2) cells or suppressor T cells (Fig. 2*B*). To determine whether the $CD4^+CD25^+$ T cells represented a T suppressor population, they were analyzed for expression of Foxp3 and TGFβ. The results demonstrated that ≥75% of $CD4^+CD25^+$ T cells were positive for Foxp3 as well as TGFβ, thus confirming their suppressor phenotype. In contrast, $CD4^+CD25^-$ cells did not express significant levels of either protein. To determine whether tumor-associated $CD4^+CD25^+$ T cells were functionally suppressive, their activity was tested in a coculture assay (Fig. 2*C*). Tumor-associated $CD4^+CD25^+$ T cells effectively suppressed the proliferation of responder T cells, whereas the $CD4^+CD25^-$ subset did not. Collectively, the above data establish that advanced tumors were infiltrated by three major T cell subsets. These included $CD8^+$ T cells that displayed an effector/memory phenotype and impaired cytolytic function, a $CD4^+CD25^-$ subset that primarily showed effector/memory Th1 characteristics and a $CD4^+CD25^+$ subset that displayed T suppressor phenotype and function.

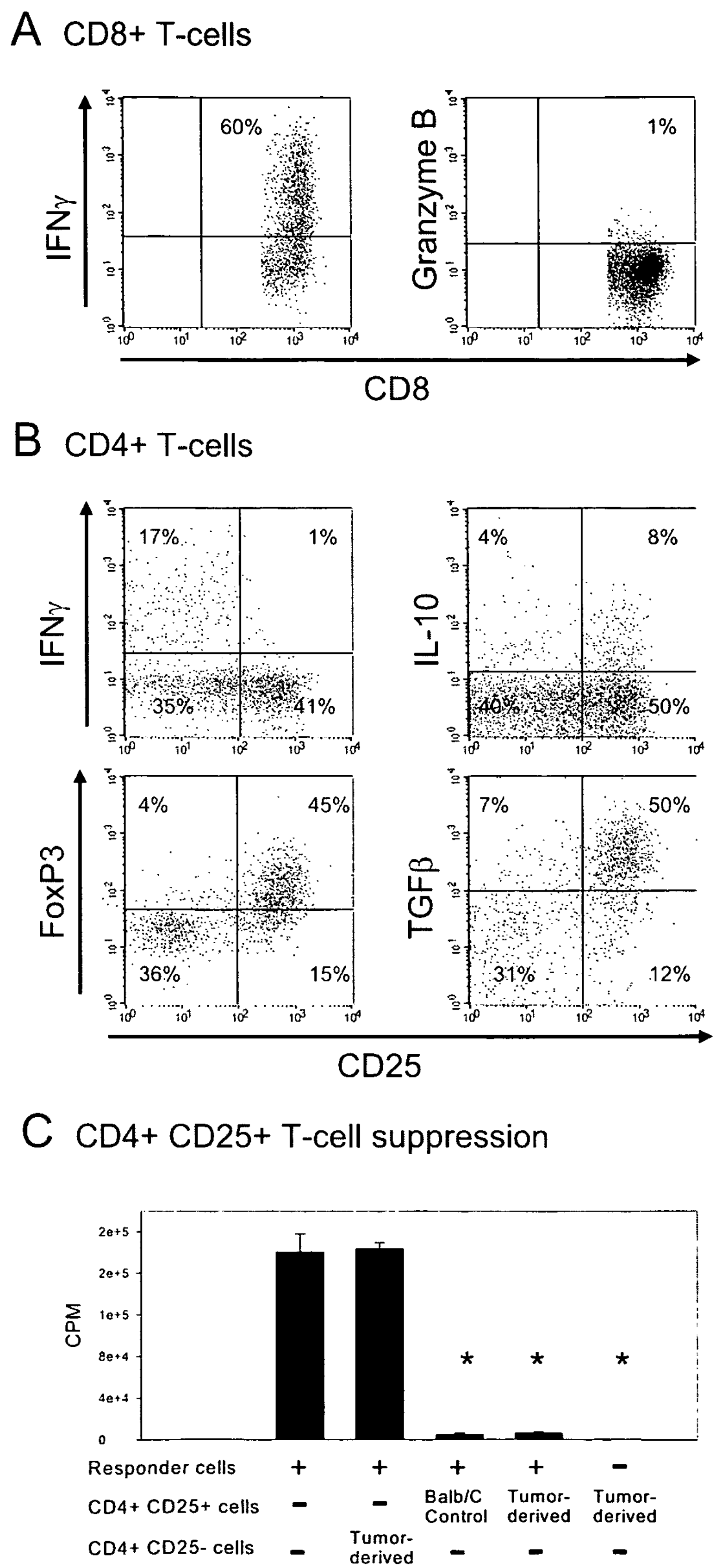

FIGURE 2. Functional analysis of tumor-infiltrating $CD8^+$ and $CD4^+$ T cells. *A*, IFN-γ and granzyme B production by $CD8^+$ T cells. TIL were stimulated with PMA/ionomycin, and $CD8^+$ T cells were gated on and analyzed for intracellular IFN-γ. Granzyme B production was analyzed by direct ex vivo staining of TIL for intracellular granzyme B. *B*, IFN-γ, IL-10, Foxp3, and TGFβ expression by $CD4^+$ T cells. All cells were stained for membrane CD4 and CD25. They were then stained for intracellular IFN-γ, IL-10, membrane-bound TGFβ, or intracellular Foxp3 as described in *Materials and Methods*. $CD4^+$ T cells were gated on and analyzed. *C*, In vitro T cell suppression assay. The ability of tumor-derived $CD4^+CD25^+$ T cells to suppress the proliferation of naive $CD4^+CD25^-$ responder cells was determined as described in *Materials and Methods*. *, The differences between responder cells alone and groups including $CD4^+CD25^+$ cells were highly significant ($p \leq 0.00006$). Error bars, SD.

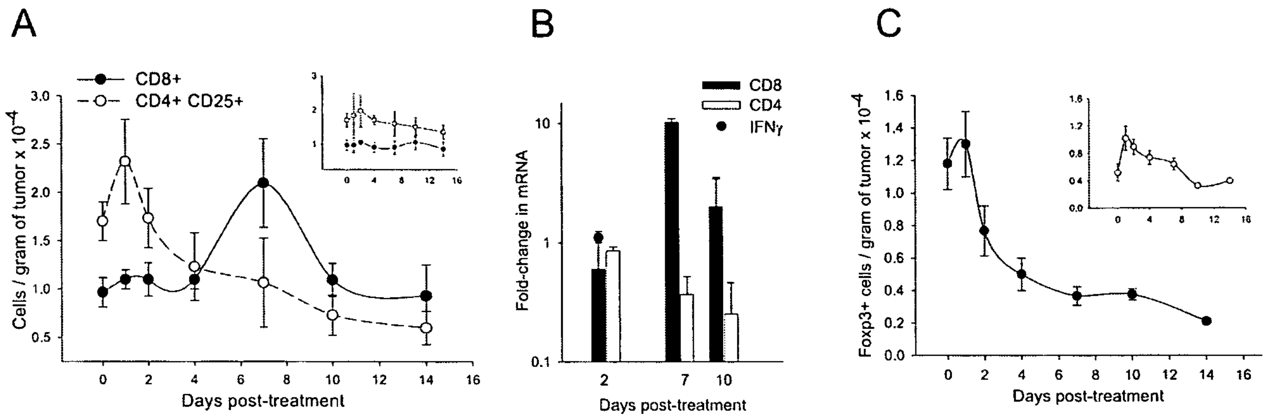

FIGURE 3. Posttherapy intratumoral T cell kinetics in the Line-1/BALB/c tumor model. *A*, Absolute numbers of tumor-infiltrating $CD8^+$ and $CD4^+CD25^+$ T cells. Following Ab staining, cells within the mononuclear lymphocyte gate were analyzed on day 0 (before therapy) and on days 1, 2, 4, 7, 10, and 14 (posttherapy). The *inset* demonstrates the cell numbers following treatment with control microspheres. Number of cells per tumor was determined by using the following formula: N × R1/T, where N = percentage of positive staining cells, R1 = total number of cells in the lymphocyte gate, and T = tumor weight in g. For $CD8^+$ T cells, $p \leq 0.025$ between day 7 and all other days. For $CD4^+CD25^+$ cells, $p \leq 0.019$ between day 1 and days 4, 7, 10, or 14. There were no statistically significant differences between any of the time points in control-treated mice (*inset*). Error bars = SD. *B*, Real-time PCR quantification of CD8, CD4, and IFN-γ. RT-PCR was performed with RNA purified from tumor FNA samples as described in *Materials and Methods*. Fold-change is relative to day 0. The differences between days 2 and 7 were significant for CD8 ($p = 0.0004$) and CD4 ($p = 0.027$). Error bars = SD. *C*, Absolute numbers of tumor-infiltrating $Foxp3^+CD4^+CD25^+$ T cells after treatment. The differences between day 0 and days 2, 4, 7, 10, or 14 were significant ($p \leq 0.03$). The *inset* demonstrates the numbers of Foxp3-negative $CD4^+CD25^+$ Th cells. All data shown in *A–C* represent combined results from three independent experiments; n = 3/experiment. Error bars, SD.

In situ vaccination with IL-12/GM-CSF microspheres induces T effector/memory activation, T suppressor purge, and $CD8^+$ T effector cell infiltration

The next series of experiments were designed to address how delivery of IL-12 and GM-CSF affected the quantity, phenotype, and function of tumor-associated T cells. Initially, quantitative analysis of T cell subsets was performed to establish the posttreatment T cell kinetics. For this purpose, established tumors were injected either with control (blank) or IL-12/GM-CSF-loaded microspheres, and the numbers of tumor-infiltrating $CD8^+$ and $CD4^+CD25^+$ T cells were monitored for a 2-wk period. The results are summarized in Fig. 3. Intratumoral T cell numbers remained unchanged after treatment with control microspheres (Fig. 3*A*, *inset*). In contrast, IL-12/GM-CSF microsphere treatment induced significant quantitative changes in both $CD8^+$ and $CD4^+CD25^+$ T cell populations (Fig. 3*A*). The most dramatic changes involved a 3-fold decrease in the number of tumor-associated $CD4^+CD25^+$ T cells between days 0 and 14 and a 2.5-fold increase in the number of $CD8^+$ T cells on day 7. Although the decrease in $CD4^+CD25^+$ T cells was progressive after day 1, there was no significant change in the number of tumor-infiltrating $CD8^+$ T cells during the first 4 days. The transient increase in $CD4^+CD25^+$ T cells on day 1 was most likely due to $CD4^+CD25^-$ Th cell activation because the number of $CD4^+CD25^-$ Th cells decreased by 1.8-fold between days 0–2 and remained unchanged thereafter (data not shown). The increase in the quantity of intratumoral $CD8^+$ T cells on day 7 and the progressive decline in $CD4^+$ T cell numbers were confirmed by real-time PCR-based quantification of CD8 and CD4 mRNAs following serial FNA of tumors (Fig. 3*B*). These data demonstrate that whereas the levels of CD8 and CD4 remained constant between days 0 and 2, CD8 transcript levels increased by almost 10-fold on day 7 and intratumoral CD4 mRNA levels decreased progressively, up to 4-fold by day 10. Intratumoral IFN-γ levels followed a pattern similar to that observed for $CD8^+$ T cells, suggesting that treatment induced a $CD8^+$ T cell-associated production of IFN-γ in vivo. Overall, CD8/CD4 kinetics obtained by real-time PCR analysis confirmed the flow cytometry data.

Whereas the data shown in Fig. 3*A* established that $CD4^+CD25^+$ T cell numbers declined between days 0 and 14, it was not clear whether this decline was specifically associated with T suppressors because the $CD4^+CD25^+$ T cell population can include both $Foxp3^+$ T suppressors and Foxp3- activated Th. To determine whether the reduction in $CD4^+CD25^+$ T cells reflected a loss of bona fide T suppressors, $Foxp3^+CD4^+CD25^+$ T cells were quantified following intracellular staining. The results shown in Fig. 3*C* confirm that treatment induced a rapid, progressive loss of T suppressor cells from the tumor, with the absolute numbers of $CD4^+CD25^+Foxp3^+$ cells decreasing by 6-fold between days 1 and 14. At the same time, analysis of the $CD4^+CD25^+Foxp3^-$ Th cells demonstrated that treatment induced a brief but significant increase in the numbers of activated Th cells between days 0 and 1 (most likely due to the activation of pre-existing $CD4^+CD25^-$ Th cells), which did not persist beyond day 2 (Fig. 3*D*, *inset*). The overall pattern that emerges from this analysis is a rapid and effective reversal of the immune-suppressive characteristics of the tumor microenvironment, which is then maintained for at least 2 wk.

IL-12/GM-CSF delivery restores effector function to tumor-associated $CD8^+$ T effector/memory cells

Phenotypic and functional analyses of CD8 and CD4 T cell populations were then performed to determine whether the observed quantitative changes correlated with functional activation. These studies demonstrated that following treatment, $CD8^+$ T cells rapidly up-regulated CD43 and CD69 expression, with the proportion of CD43/CD69 double-positive cells increasing from an average of 17% on day 0 to 60% on day 2 and then to 65% on day 4 (Fig. 4, *A* and *C*). Because there was no increase in cell numbers between days 0 and 4, these findings supported the notion that treatment induced a rapid conversion of pre-existing quiescent $CD8^+$ T

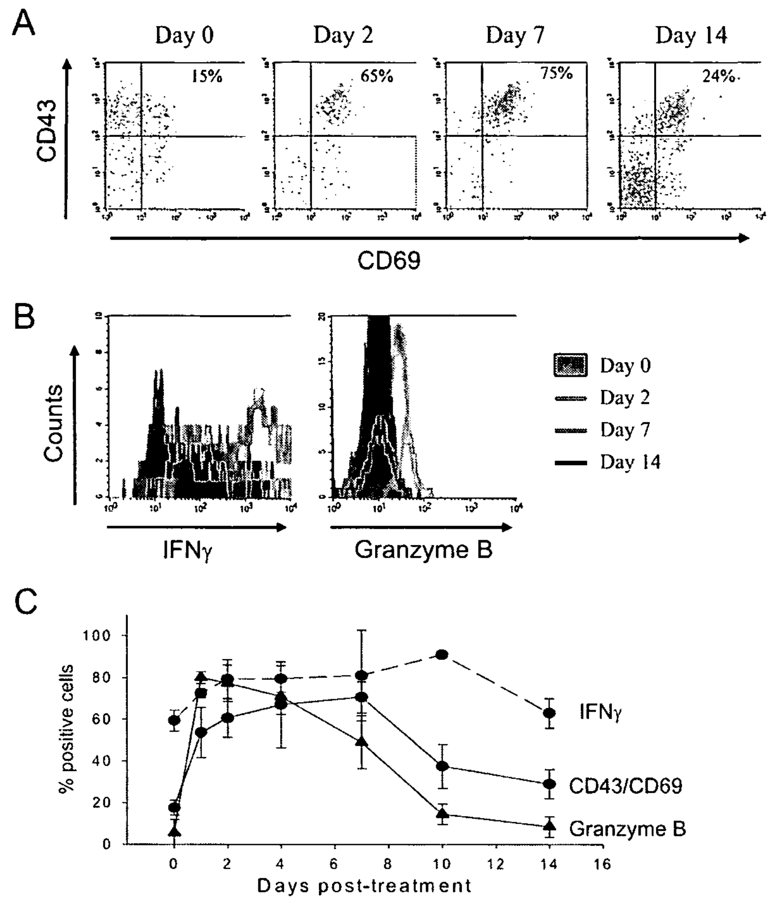

FIGURE 4. Functional analysis of posttherapy $CD8^+$ T cells. *A*, Expression of CD43 and CD69. Single-cell suspensions prepared from tumors were stained for CD8, CD43, and CD69. $CD8^+$ T cells were gated on and analyzed for CD43 and CD69 expression on days 0, 2, 7, and 14. Data shown are representative of three independent experiments. *B*, $CD8^+$ T cells were stained for intracellular expression of IFN-γ and granzyme B. Representative results for days 0, 2, 7, and 14 are shown. *C*, Comprehensive analysis of posttreatment $CD8^+$ T cell activation kinetics. Percentage of cells positive for CD43/CD69, IFN-γ, and granzyme B are shown (days 0–14). Each point is an average of three independent experiments; $n = 3$/experiment. For all three markers, the differences between day 0 and days 1–7 were significant ($p \leq 0.044$). Error bars, SD.

effector/memory cells to activated effector cells in the absence of proliferation. The activation level of day 7 $CD8^+$ T cells was similar to that of day 4 cells with 70% of $CD8^+$ T cells demonstrating a CD43/CD69 double-positive phenotype. In contrast to the day 2 cells, however, there was a dramatic increase in the number of $CD8^+$ T cells on day 7, suggesting that day 7 $CD8^+$ T effectors were distinct from day 2 cells (Fig. 3*A*). Both the activation level (Fig. 4, *A* and C) and the absolute number (Fig. 3*A*) of T effectors declined after day 7, approaching pretherapy levels by day 14.

Functional analysis of intratumoral $CD8^+$ T cells demonstrated that treatment enhanced both the proportion of IFN-γ-secreting cells (from an average of 58 to 80%) and the amount of IFN-γ produced per cell (average mean fluorescence intensity increasing from 230 + 30 to 510 + 60) between days 0 and 2 (Fig. 4, *B* and *C*). More importantly, the cytotoxic activity of $CD8^+$ T cells showed a dramatic change, with the percentage of granzyme B-positive cells increasing from 5% on day 0 to 80% on day 2 (Fig. 4, *B* and *C*). The activated effector characteristics of $CD8^+$ T cells were maintained through day 7 with regard to both IFN-γ production and granzyme B expression (Fig. 4*C*), except that on a per cell basis, day 7 cells demonstrated much higher production of IFN-γ compared with day 2 cells (average mean fluorescence intensity of 510 ± 60 vs 1300 ± 200 on days 2 and 7, respectively). This finding again suggested that day 7 $CD8^+$ T cells were functionally different from day 2 cells. By day 14, $CD8^+$ T cell activity decreased significantly with the proportion of IFN-γ and granzyme B-positive cells retreating to pretherapy levels.

Treatment-induced activation of $CD8^+$ T effector/memory cells is followed by rapid apoptotic cell death, the pattern of which supports two different phases of $CD8^+$ T cell activity within the tumor microenvironment

The above studies demonstrated that conversion of pre-existing $CD8^+$ T effector/memory to a T effector phenotype on day 2 was followed by the expansion of the $CD8^+$ T effector cell population on day 7. However, it was not clear whether day 7 expansion was due to delayed proliferation of pre-existing T cells or involved the infiltration of tumor with a distinct, exogenous $CD8^+$ effector T cell population, possibly migrating from the TDLN. $CD8^+$ TIL with a quiescent nonapoptotic phenotype have been shown to apoptose rapidly after activation due to activation-induced cell death (AICD) (23, 24), a finding that is inconsistent with the notion that treatment resulted in the proliferation of pre-existing $CD8^+$ T effector/memory cells. We thus hypothesized that analysis of posttherapy $CD8^+$ T cell apoptosis may provide additional clues as to whether day 2 and day 7 T effector populations were distinct. To this end, the apoptotic profile of tumor-associated $CD8^+$ T cells was determined between days 0–7. The results established that the percentage of Annexin V^+CD8^+ T cells increased rapidly from 8% on day 0 to 45% on day 4 following IL-12/GM-CSF delivery, demonstrating the induction of apoptosis (Fig. 5*A*). Further analysis of downstream apoptotic events, i.e., expression of antiapoptotic molecules Bcl-x_L/Bcl-2 and activation of proapoptotic caspase-3, demonstrated that whereas the expression of antiapoptotic proteins remained stable between days 0 and 4 (except for a

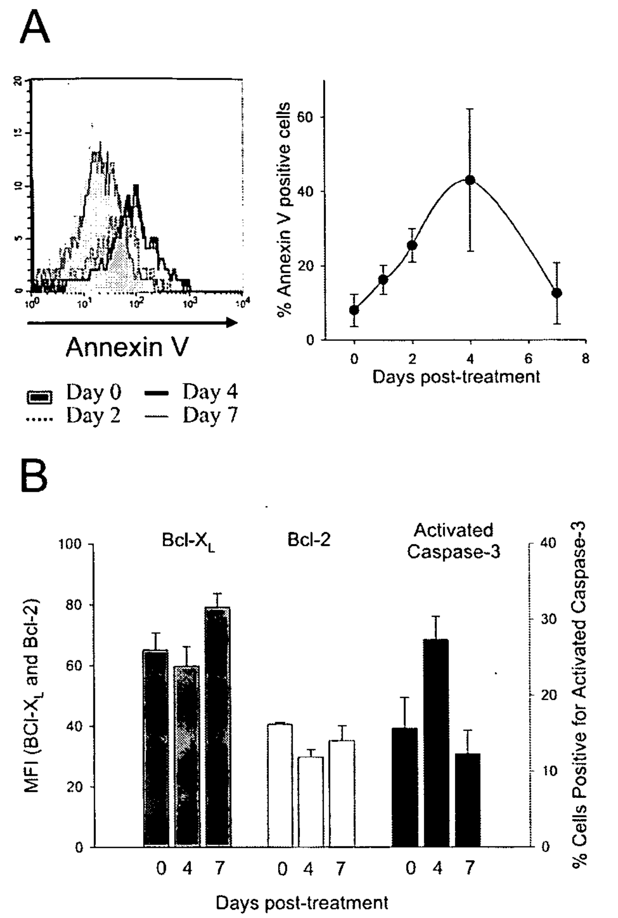

FIGURE 5. Posttherapy CD8$^+$ T cell apoptosis. *A*, Annexin V binding. CD8$^+$ T cells were stained for Annexin V binding. Annexin V$^+$ gate was determined based on Annexin V$^-$ staining of naive LN lymphocytes (>98% live; data not shown). The line graph demonstrates average percentage of Annexin V-positive cells from three independent experiments (n = 3/experiment). The differences between day 4 and days 1, 2, or 7 were significant ($p \leq 0.037$). Error bars, SD. *B*, Bcl-x_L/Bcl-2 expression and caspase-3 activation. CD8$^+$ T cells were stained for intracellular Bcl-2, Bcl-x_L, and activated caspase-3 as described in *Materials and Methods* (n = 3/group). The increase in Bcl-x_L expression between days 4 and 7 was significant ($p = 0.013$). For caspase-3, the differences between day 4 and days 0 or 7 were significant ($p \leq 0.027$). Error bars, SD.

slight decrease in Bcl-2), activated caspase-3 levels increased significantly (Fig. 5*B*). The increase in caspase-3 activity on day 4 confirmed the Annexin V data and further supported the notion that CD8$^+$ T effector/memory activation was followed by irreversible apoptotic cell death. In addition, enhancement of caspase-3 activity in the absence of a substantial decline in Bcl-x_L/Bcl-2 levels suggested a primary role for the extrinsic death-receptor pathway in CD8$^+$ T effector/memory cell apoptosis consistent with AICD (25, 26). In contrast, analysis of day 7 CD8$^+$ T effectors showed a dramatic reversal in their apoptotic phenotype with both the proportion of Annexin V$^+$ and activated caspase-3$^+$ cells declining by 3.5- and 2.5-fold, respectively (Fig. 5, *A* and *B*). In addition, a slight but significant increase in the level of Bcl-x_L was observed on day 7 (Fig. 5*B*). The induction of CD8$^+$ T cell apoptosis between days 0–4, combined with the sudden reversal in their apoptotic profile after day 4 suggested that day 7 T effectors were distinct from the original T effector/memory cells and likely represented a new population.

Loss of CD4$^+$CD25$^+$ T suppressor cells from the tumor microenvironment is due to apoptotic cell death

Because treatment resulted in the rapid apoptosis of pre-existing CD8$^+$ T effector/memory cells, we hypothesized that loss of CD4$^+$CD25$^+$ T suppressors from the tumor microenvironment could also be due to apoptotic cell death. To this end, CD4$^+$CD25$^+$ T cells were evaluated for Annexin V binding (Fig. 6*A*). The results demonstrate a significant and progressive increase in the proportion of Annexin V$^+$CD4$^+$CD25$^+$ cells between days 0 and 4 (from an average of 18 to 45%), establishing that treatment induced CD4$^+$CD25$^+$ T cell apoptosis. Cell death declined between days 4 and 7 and leveled off thereafter, a pattern that correlated well with the kinetics of Foxp3$^+$ T cell loss from the tumor (Fig. 6*A*). The possibility that apoptosis was associated with activated CD4$^+$CD25$^+$ Th cells rather than bona fide T suppressors was ruled out by further analysis of CD4$^+$CD25$^+$TGFβ^+ T suppressors for Annexin V-binding, which demonstrated that treatment specifically induced apoptosis within this subset (data not shown).

Analysis of activated caspase-3 levels in posttherapy CD4$^+$CD25$^+$ T cells established that treatment induced caspase-3 activation between days 0–4, which then subsided on day 7, consistent with the Annexin V data. Similar to that observed with CD8$^+$ T cells, Bcl-x_L/Bcl-2 levels did not change between days 0–4 (Fig. 6*B*). In contrast, Bcl-x_L/Bcl-2 levels declined between days 4 and 7 in CD4$^+$CD25$^+$ cells, suggesting a partial involvement of the intrinsic mitochondrial pathway during later stages of apoptosis in CD4$^+$CD25$^+$ T cells.

Treatment induces the up-regulation of Fas ligand (FasL) expression on CD8$^+$ T effector/memory and CD4$^+$CD25$^-$ Th cells

Posttherapy T cell apoptosis was not simply a byproduct of enhanced cytotoxic activity within the tumor microenvironment because analysis of other leukocyte subsets, i.e., monocytes and granulocytes, did not show increased apoptosis between days 0–4 (data not shown). Both the activation kinetics and the pro-/anti-apoptotic protein expression patterns of intratumoral CD8$^+$ T cells suggested that the observed events were more consistent with death receptor-mediated AICD (25, 26). To this end, membrane expression of Fas and FasL on the T effector/memory and T suppressor subsets was monitored before and after therapy. The results are shown in Fig. 6*C*. Analysis of pretreatment cells demonstrated that whereas all three T cell subsets expressed Fas, none expressed FasL. Upon treatment, Fas expression did not change significantly (data not shown). In contrast, FasL expression increased on CD8$^+$ and CD4$^+$CD25$^-$ T effector/memory cells but not on CD4$^+$CD25$^+$ T suppressors on day 4 (Fig. 6*D*). Analysis of the cells on day 7 showed that FasL expression on CD8$^+$ T cells decreased back to day 0 levels, a finding consistent with the notion that day 7 CD8$^+$ T cells are distinct from the day 0–4 populations. A similar change was not observed for the CD4$^+$CD25$^-$ cells that remained FasL positive. No change was observed for the CD4$^+$CD25$^+$ T suppressors that remained FasL negative throughout (Fig. 6*D*). These data are consistent with the hypothesis that posttherapy apoptosis of tumor-associated T effector/memory cells was likely due to Fas/FasL-mediated AICD.

Both CD8$^+$ T effector cell activation and T suppressor apoptosis are mediated by IL-12 in an IFN-γ-dependent manner

The above studies were performed with IL-12/GM-CSF-encapsulated microspheres because these cytokines were previously shown to be synergistic in the long-term eradication of metastatic lung tumors in the Line-1-BALB/c model (14). It was of interest to

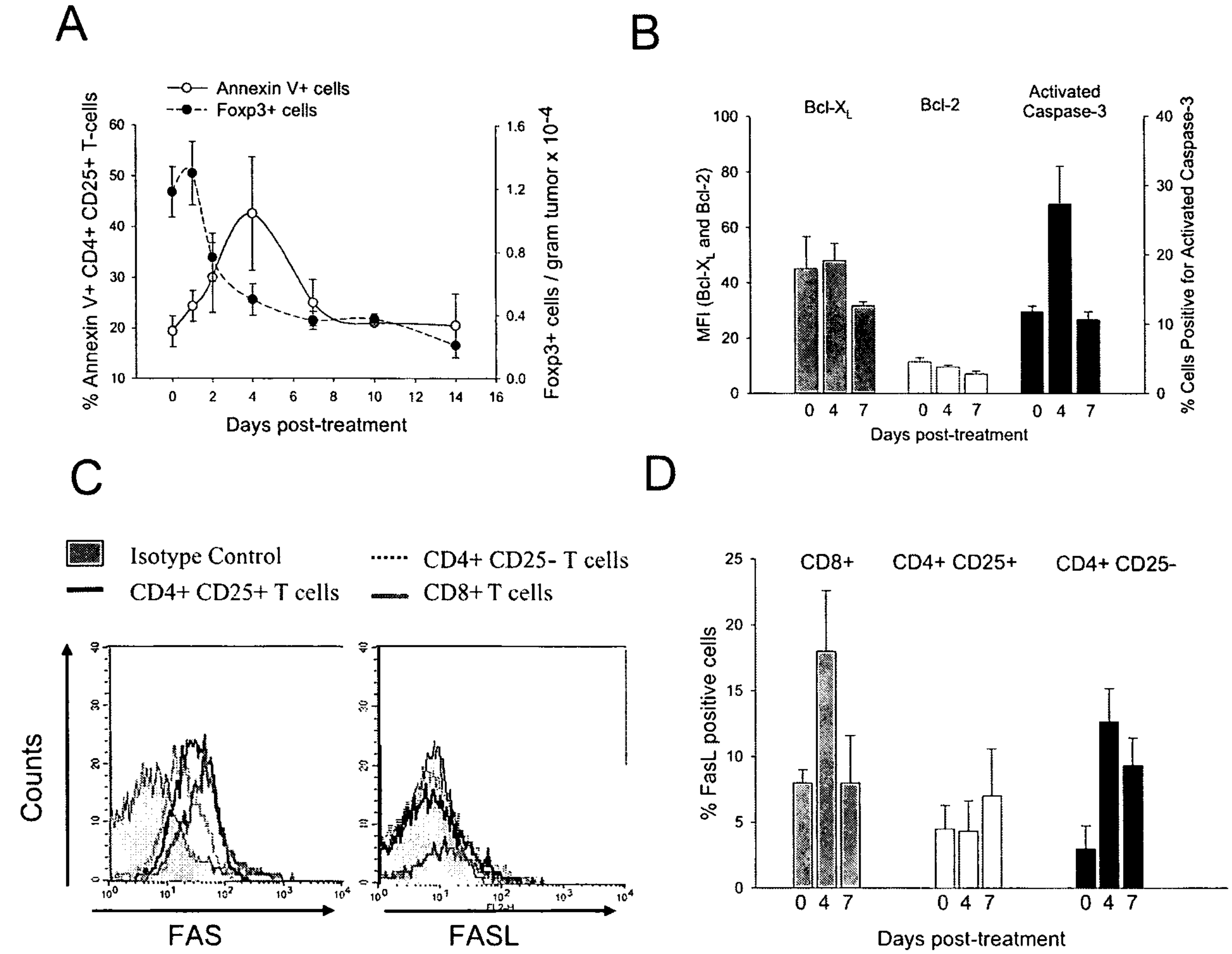

FIGURE 6. Posttherapy CD4$^+$CD25$^+$ T suppressor cell apoptosis and Fas/FasL expression. *A*, CD4$^+$CD25$^+$ T cell apoptosis. Pretreatment (day 0) and posttreatment (days 1, 2, 4, 7, 10, and 14) TIL were analyzed. Cells were stained for CD4, CD25, and Annexin V. CD4$^+$CD25$^+$ cells were gated on and percentage of Annexin V-positive cells were determined (*left ordinate*). Each point is an average of three independent experiments (n = 3/experiment). The differences between day 4 and days 1, 2, or 7 were significant ($p \leq 0.05$). Quantitative analysis of intratumoral CD4$^+$CD25$^+$Foxp3$^+$ cells as shown in Fig. 3*D* is superimposed to demonstrate the relationship between apoptosis and cell numbers (*right ordinate*). Error bars = SD. *B*, Bcl-x$_L$/Bcl-2 expression and caspase-3 activation. CD4$^+$CD25$^+$ T cells were stained for intracellular Bcl-2, Bcl-x$_L$, and activated caspase-3 as described in *Materials and Methods* (n = 3/group). The decreases observed for Bcl-2 and Bcl-x$_L$ between day 4 and 7 were significant ($p \leq 0.014$). The differences between day 4 and days 0 or 7 for caspase-3 were significant ($p \leq 0.006$). Error bars, SD. *C*, Fas and FasL expression on tumor-infiltrating T cells. Cells from day 0 tumors were stained for CD4, CD25, Fas, and FasL, or CD8, Fas, and FasL. CD8$^+$, CD4$^+$/CD25$^+$ or CD4$^+$/CD25$^-$ cells were gated on and analyzed for Fas/FasL expression. Results are representative of two independent experiments; n = 3/experiment. *D*, Posttherapy FasL expression. Tumor-infiltrating T cell subsets (CD8$^+$, CD4$^+$CD25$^+$, and CD4$^+$CD25$^-$) were stained for FasL as described above on days 0, 4, and 7, and percentage of FasL-positive cells were determined. Each bar is an average of three independent experiments, with n = 3/experiment. The differences between days 0 and 4 were significant for CD8$^+$ and CD4$^+$CD25$^-$ cells ($p \leq 0.021$). Error bars, SD.

determine the specific contribution of each cytokine to the therapy-induced changes in tumor-associated T cell subsets. To this end, we compared the effect of IL-12 and GM-CSF administered individually or together on T effector/memory cell activation, T suppressor cell loss, and T effector cell infiltration. The results are shown in Fig. 7. These data demonstrate that both the initial CD8$^+$ T effector/memory cell activation as determined by CD43/CD69 expression, as well as the increase in CD8$^+$ T effector cell numbers on day 7 were primarily mediated by IL-12 and not GM-CSF (Fig. 7, *A* and *B*). Similarly, posttherapy T suppressor apoptosis (Fig. 7, *C* and D) and up-regulation of FasL on CD8$^+$ T cells (Fig. 7*E*) were also dependent on IL-12 and not GM-CSF.

Numerous studies, including ours, have demonstrated that IL-12-mediated tumor regression is IFN-γ dependent (14, 15). Others showed that IFN-γ is also required for death receptor-mediated apoptosis (27–30). This raised the question whether the CD8$^+$ T effector cell activation and the CD4$^+$CD25$^+$ T suppressor cell apoptosis observed in this study were mediated directly by IL-12 or required the induction of downstream effectors, i.e., IFN-γ. To this end, therapy-induced changes in tumor-infiltrating CD8$^+$ and CD4$^+$CD25$^+$ T cells were monitored in wild-type (wt) and IFN-γ-knockout (GKO) mice. The results are shown in Table I. These data demonstrate that both CD8$^+$ T effector cell expansion and CD4$^+$CD25$^+$ T suppressor cell loss/apoptosis required IFN-γ. We therefore conclude that IFN-γ is central to the IL-12-induced changes in intratumoral T cell populations.

Discussion

The studies described here establish that delivery of IL-12 to tumors promotes an effective reversal of tumor immune suppression via the activation of tumor-associated T effector/memory cells, the elimination of intratumoral CD4$^+$CD25$^+$ T suppressor cells, and

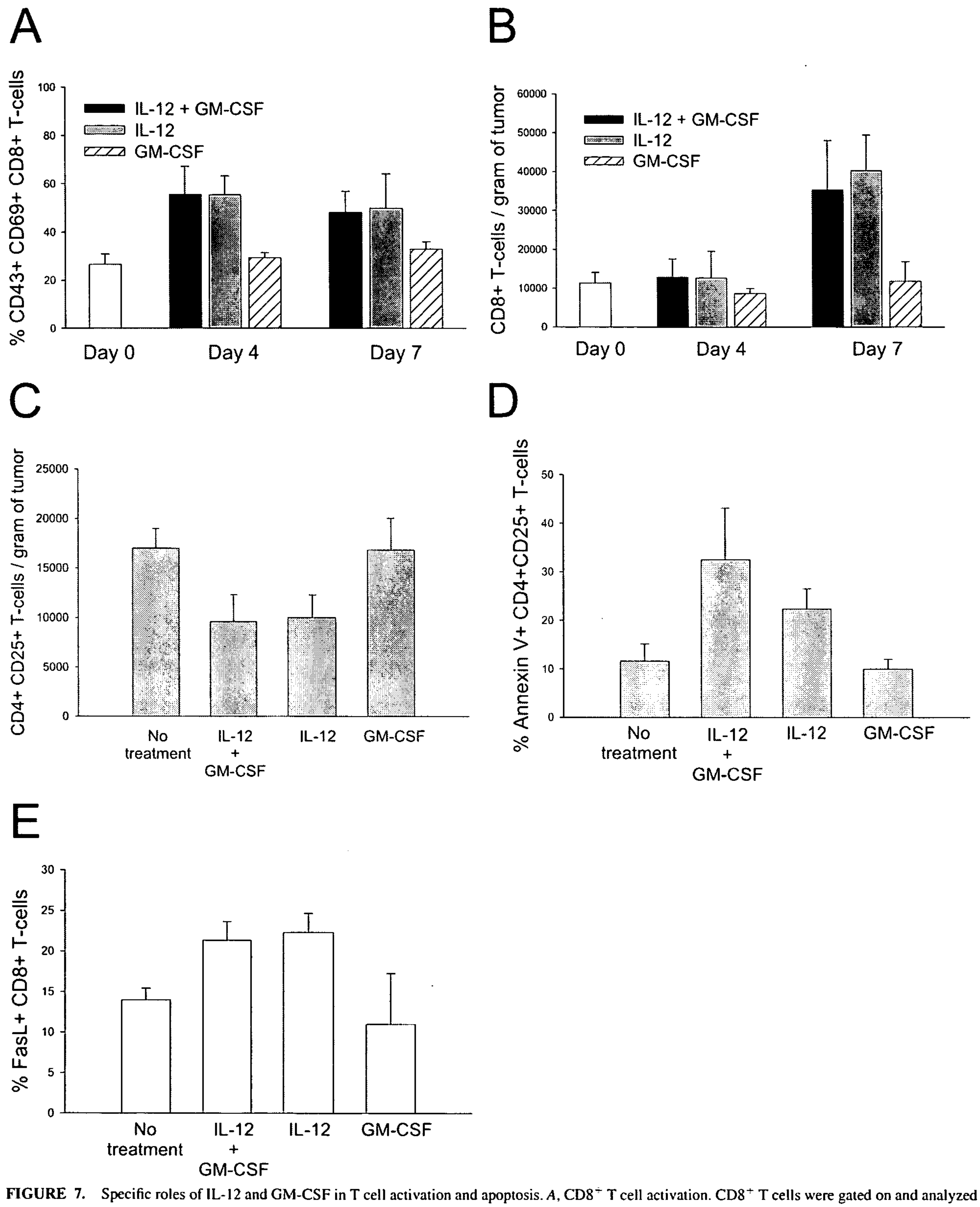

FIGURE 7. Specific roles of IL-12 and GM-CSF in T cell activation and apoptosis. *A*, $CD8^+$ T cell activation. $CD8^+$ T cells were gated on and analyzed for double-positive (CD43/CD69) cells on days 0 (untreated tumor) or days 4 and 7 following treatment with IL-12, GM-CSF, or IL-12 + GM-CSF microspheres (n = 3/group). The differences between day 0 and days 4 or 7 were significant ($p \leq 0.05$) for the IL-12 + GM-CSF or IL-12 alone groups. The differences between day 0 and days 4 or 7 were not significant ($p \geq 0.10$) for GM-CSF alone. Error bars, SD. *B*, $CD8^+$ T cell proliferation kinetics. Absolute numbers of $CD8^-$ T cells in tumors were determined on day 0 (untreated tumor) or days 4 and 7 following treatment with IL-12, GM-CSF, or IL-12 + GM-CSF microspheres (n = 3/group). The differences between day 0 and day 7 were significant ($p \leq 0.03$) for IL-12 + GM-CSF or IL-12 alone. The difference between day 0 and day 7 was not significant ($p = 0.57$) for GM-CSF alone. Error bars, SD. *C*, T suppressor cell kinetics. Absolute numbers of tumor-infiltrating $CD4^+CD25^+$ T cells were determined on day 0 (no treatment) and 4 days after treatment in each group (n = 3/group). The differences between no treatment and IL-12 + GM-CSF or IL-12 alone groups were significant ($p \leq 0.019$). The difference between no treatment and GM-CSF alone

Table I. *IFN-γ is required for IL-12-induced T effector cell expansion and T suppressor cell apoptosis*[a]

	$CD8^+$ T Cells		$CD4^+CD25^+$ T Suppressor Cells			
	Cells/g tumor × 10^{-3}[b]		Cells/g tumor × 10^{-3}[c]		Percentage of caspase-3-positive cells[c]	
Day	wt	GKO	wt	GKO	wt	GKO
0	33.3 ± 3.5	18.1 ± 3.4	52.1 ± 8.3	66 ± 19	10.5 ± 2.1	10.5 ± 0.7
4	29 ± 3.5	12.8 ± 1.8	13.5 ± 1.2	63.5 ± 7.2	22.3 ± 3.6	9.8 ± 0.9
7	83.5 ± 11.2	11.6 ± 1.7	ND	ND	ND	ND

[a] Values shown are averages of three mice per group. Error, SD. ND, Not done.
[b] The differences between day 7 and days 0/4 were significant in wt mice ($p \leq 0.002$).
[c] The differences in cell numbers and caspase-3 activity between days 0–4 were significant in wt mice ($p \leq 0.015$).

the infiltration of $CD8^+$ T effectors. This reversal is maintained for at least 2 wk after treatment due to continued loss of $CD4^+CD25^+$ T suppressors from the tumor. These data demonstrate the highly complex nature of post-IL-12 therapy T cell activity in the tumor microenvironment and provide a detailed understanding of how intratumoral delivery of IL-12 microspheres promotes tumor regression.

Characterization of tumor-associated T lymphocytes before treatment identified three distinct T cell subsets. These subsets included functionally impaired $CD8^+$ and $CD4^+CD25^-$ T cells with an effector/memory phenotype, and a $CD4^+CD25^+Foxp3^+$ $TGF\beta^+$ T cell population that demonstrated suppressive function. These data are consistent with the findings of previous studies demonstrating the quiescent nature of tumor-associated $CD8^+/CD4^+$ T cells (6, 31–33), and more recently the presence of T suppressor cell infiltrates in murine and human tumors (34). Evidence from other studies suggests that the dysfunctional state of tumor-associated T cells is, at least in part, due to T suppressor activity, establishing a functional link between the two populations (7–9). To this end, it was recently demonstrated that in vivo, T suppressor cells modulate antitumor T effector activity via selective inhibition of cytotoxic function, and that TGFβ is critical to inhibition (35). Our findings that pretherapy T effector/memory cells were impaired in granzyme B production, and that the tumor-infiltrating $CD4^+CD25^+$ T suppressor cells expressed membrane-bound TGFβ, are consistent with the notion that T suppressor cells are responsible for T effector/memory cell dysfunction in our model.

Intratumoral delivery of IL-12 resulted in a rapid and dramatic reversal of the anergic phenotype and function of pre-existing T effector/memory cells. Several studies have shown that whereas TIL are dysfunctional in situ, purified $CD8^+$ T cells can be reactivated in vitro upon stimulation with cytokines (6). This study demonstrates a highly effective rescue of both the phenotypic and functional characteristics of tumor-associated T cells in situ. Others recently reported that local delivery of IL-12 induced the activation of tumor-associated $CD4^+$ T cells, resulting in an IFN-γ-dependent eradication of tumor xenografts in a human tumor/SCID mouse xenograft model (36). Our data establish that treatment resulted in the activation of both $CD4^+$ and $CD8^+$ T effector/memory cells and that in addition to enhanced IFN-γ production, a concurrent recovery of $CD8^+$ T cell cytolytic function was achieved. More importantly, we also found that activation of pre-existing $CD8^+$ T effector/memory cells did not result in their proliferation, but led to apoptotic death within 4 days of treatment. This finding is consistent with others' observations that anergic, tumor-associated T cells are predisposed to AICD upon in vitro activation (23) and that IL-12-mediated activation enhances Fas/FasL-dependent AICD of T cells (37, 38). To this end, analysis of FasL expression on tumor-associated T effector/memory cells in our model demonstrated that treatment resulted in the up-regulation of FasL on $CD8^+$ T cells and $CD4^+$ Th cells, consistent with the onset of AICD.

An unexpected finding in this study was that, concurrent with the activation of $CD8^+$ T effector/memory cells, intratumoral delivery of IL-12 induced a rapid quantitative decline in tumor-infiltrating T suppressor cells. Further analysis established that the loss of T suppressors from tumors was due to apoptotic cell death, which was detectable within 24 h of treatment and peaked on day 4 posttherapy. $CD4^+CD25^+$ T cells displayed Fas on their cell surface, suggesting that the Fas/FasL pathway could be involved in therapy-induced apoptosis. Because treatment did not induce FasL expression on T suppressors, the death signal (FasL) was likely provided by activated T effector/memory cells. Current evidence for the role of Fas/FasL-mediated apoptosis in T suppressor homeostasis is inconclusive. Although T suppressor cells express Fas constitutively (39, 40), they are resistant to anti-Fas Ab-mediated apoptosis (41). In contrast, it was recently reported that whereas $CD4^+CD25^+$ T suppressors were resistant to AICD via TCR-stimulation, they were uniquely sensitive to soluble CD95 ligand-mediated apoptosis in vitro (42). Similarly, overexpression of Foxp3 in $CD4^+CD25^+$ T cells of Foxp3 transgenic mice has been shown to result in hypersensitivity to Fas/FasL-mediated apoptosis (43). These studies support the hypothesis that the Fas/FasL pathway may be involved in T suppressor homeostasis. Whether elimination of T suppressor cells from immunologically active environments via death receptor-mediated apoptosis is a general mechanism for overcoming regulation remains to be shown. In this case, T suppressor loss from tumors continued for at least 2 wk, providing a relatively broad window for the effector responses to occur. Whether T suppressor cells eventually reinfiltrated persisting tumors was not determined.

Activation and subsequent apoptotic death of pre-existing $CD8^+$ T effector/memory cells between days 0–4 was followed by infiltration of tumors with nonapoptotic $CD8^+$ T cells displaying full effector phenotype on day 7. Apoptosis did not result in a

was not significant ($p = 0.94$). Error bars = SD. *D*, T suppressor cell apoptosis. Annexin $V^+CD4^+CD25^+$ T cells were quantified on day 0 (no treatment) and 4 days after treatment in each group ($n = 3$/group). The differences between untreated mice and the IL-12 + GM-CSF or IL-12 alone groups were significant ($p \leq 0.04$). The difference between no treatment and GM-CSF alone was not significant ($p = 0.51$). Error bars, SD. *E*, $CD8^+$ T cell FasL expression. $CD8^+$ T cells were gated on and analyzed for FasL expression on day 0 (no treatment) or on day 4 after treatment for each group ($n = 3$/group). The differences between no treatment and IL-12 + GM-CSF or IL-12 alone groups were significant ($p \leq 0.03$). The difference between no treatment and GM-CSF alone was not significant ($p = 0.57$).

detectable reduction in intratumoral $CD8^+$ T cell numbers between days 0–4 (as seen with T suppressor cells), due possibly to the compensatory infiltration/expansion of $CD8^+$ T effectors. Whether day 7 $CD8^+$ T effectors expanded from pre-existing T effector/memory cells or represented a secondary wave of newly primed $CD8^+$ T cells arriving from the TDLN remains equivocal. The significant differences in the intensity of IFN-γ production by day 2 vs day 7 $CD8^+$ T cells, the sudden switch in the apoptotic phenotype of day 7 $CD8^+$ T cells, as well as the long interval between IL-12 delivery and the actual expansion collectively suggest that day 7 population was distinct from pre-existing $CD8^+$ T effector/memory cells and likely represented newly primed effectors arriving from the TDLN. In contrast, the current data cannot rule out the possibility that a subset of pre-existing T effector/memory cells escaped/reversed AICD and expanded on day 7. Studies addressing this possibility are currently underway. Regardless of source, the secondary T effector response contracted rapidly by day 10, consistent with the established activation kinetics of cytotoxic T cell responses (23, 44).

Analysis of TIL from mice treated either with IL-12 or GM-CSF alone demonstrated that essentially all posttreatment T cell activity described in this study was mediated by IL-12. The ability of IL-12 to directly enhance the activity and proliferation of Ag-experienced T cells is well-established (15). Therefore, the dominant role of IL-12 in mediating the early posttherapy changes within tumor-infiltrating T cell populations is not surprising. To this end, our earlier studies demonstrated synergy between these cytokines in the long-term eradication of systemic disease, but not necessarily in short-term regression of primary tumors (Ref. 14 and M. O. Kilinc and N. K. Egilmez, unpublished data). Because GM-CSF has been shown to augment tumor vaccines primarily via the induction of APC generation (16), its synergistic properties are likely associated with a qualitative enhancement of long-term T cell memory.

The observation that the secondary $CD8^+$ T effector response contracted rapidly between days 7–14, even in the absence of T suppressor cells, has important clinical implications. Although treatment resulted in an effective reversal of immune suppression within the tumor microenvironment and enhanced the intensity of T cell activity, cumulative effector activity was still transient, providing a cytotoxic window between days 1 and 10. T effector cell activation/contraction kinetics has been shown to be independent of both Ag load and persistence (44) and, as the above results suggest, possibly of T suppressor activity. Whereas manipulation of Ag dose, use of inflammatory adjuvants, and/or blocking of regulation enhances the intensity of T cell responses, the contraction kinetics of T effector cells remain unaltered (44, 45). Therefore, long-term efficacy of vaccine-based approaches may be limited by tumor burden and/or persistence even when immune regulatory mechanisms are successfully blocked. Accordingly, vaccines designed to induce antitumor T cells would be more likely to achieve complete tumor eradication and disease-free survival in patients with limited tumor burden. To this end, therapeutic tumor vaccines have been significantly more successful when administered in the minimal residual disease setting in both preclinical and clinical studies (14, 46–48).

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Disclosures

N. K. Egilmez has partial ownership in Therapyx, Incorporated, which is currently developing the microsphere technology for tumor vaccination.

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CD69 expression in B-cell chronic lymphocytic leukemia: a new prognostic marker ?

Ninety-two patients suffering from immunologically typical (CD5+CD23+) B-cell chronic lymphocytic leukemia (B-CLL) were tested for the expression of CD69, an antigen that is precociously expressed on normal stimulated T lymphocytes and B-cells. Forty-eight (52%) patients displayed CD69 antigen on the cell surface and the expression of this molecule was found to be related to higher peripheral blood lymphocytosis, more advanced clinical stage, a diffuse pattern of bone marrow infiltration, and trisomy 12. By contrast, del13q14 was more frequently detected in the CD69-negative group. Finally, CD69 expression had a significantly negative impact on survival of patients. These data suggest that CD69 could be a promising new immunologic prognostic marker for B-CLL.

A highly variable clinical course of disease characterizes B-cell chronic lymphocytic leukemia (B-CLL).[1] In fact, some patients do not require any treatment for many years and have a long-standing disease, while others may die within a few months of diagnosis because of B-CLL itself or disease-related complications. In an attempt to identify subgroups of B-CLL patients with peculiar features predictive of the clinical behavior of the disease, several clinical and laboratory parameters have already been tested.[2] Indeed, various immunophenotypic markers have been proposed as having prognostic relevance, such as the intensity of CD20 expression,[3] the expression of CD23 and CD21,[4] and, more recently, the expression of CD38.[5]

CD69 identifies a type II integral membrane protein with a sir[illegible]gle transmembrane domain belonging to the C-type lectin f[illegible]ily of surface receptors.[6,7] Initially described as an a[illegible] expressed early in the activation of lymphoid cells, C[illegible] considered restricted to activated lymphocytes. As a [illegible] of the fact, resting peripheral blood lymphoid cells do n[illegible]xpress CD69. However, the stimulation of the T-cell re[illegible]/CD3 complex in T-cells quickly induces expression of [illegible] In addition, CD69 expression is inducible by immatu[illegible]cytes, B-cells (through crosslinking of surface immu[illegible]), natural killer cells, monocytes, neutrophils and e[illegible]s. Although a specific ligand has not yet been ide[illegible], [illegible]D69 generates intracellular signals with various cel[illegible]ponses.[9]

We analyzed CD69 (clon[illegible] [illegible]cton Dickinson Immunocytometry Systems, BDIS, S[illegible]A, USA) expression on neoplastic cells by means [illegible]cytometry (FACSCalibur, BDIS) in peripheral blood sample[illegible]rom 92 immunologically typical (CD5+CD23+) untreated B-CLL patients. An additional panel of fluorescein (FITC) and phycoerythrin (PE) directly-conjugated monoclonal antibodies including CD19 (Leu-12), CD20 (Leu-16), CD22 (leu-14), CD23 (Leu-20), CD5 (Leu-1), CD38 (Leu-17), FMC7, κ/λ light chains, all purchased from BDIS, and CD79b (CB3-1, Immunotech, Marseille, France), was used. Finally, the number of CD20 and CD22 molecules/per cell, evaluated as antibody-binding capacity (ABC), was measured by means of QuantiBRITE technology (BDIS), as described elsewere.[10]

Forty-eight (52%) patients expressed CD69 in more than 30% of CD19-positive cells. Table 1 reports the clinico-biological features of B-CLL patients according to the expression of CD69. As shown, no differences by age, gender, typical or atypical morphology (FAB criteria), expression of FMC7, CD79b and CD38, as well as density of CD20, CD22 and surface membrane immunoglobulins were observed between the two groups of patients. However, peripheral blood lymphocytosis, Binet stages B and C, and diffuse pattern of bone marrow infiltration were found to be closely associated with CD69 expression. In addition, trisomy 12 (a cytogenetic marker of poor prognosis) was significantly more represented in the CD69-positive group, while del 13q14 (usually correlated with a better prognosis) was detected more frequently in the CD69-negative group. Ten patients (5 in the CD69-positive and 5 in the CD69-negative group) did not carry any of these abnormalities. As a result, median overall survival of CD69-positive B-CLL patients was 98 months, while it is still not reached at 150 months in the CD69-negative B-CLL patients (Figure 1). Finally, multivariate analysis (Cox model) confirmed the independent positive prognostic weight of CD69 expression at diagnosis in B-CLL (p = 0.015) (data not shown).

Table 1. Clinico-biological features of B-CLL patients according to the expression of CD69 molecules.

Features	*CD69 expression*		*p*
	Negative	*Positive*	
Age	62.6 ± 10,4	63.1 ± 11,8	ns°
Sex (M/F)	34/10	27/21	ns*
Morphology			
Typical	37	38	ns°
Atypical	7	10	ns°
Clinical stage (Binet)			
A	20	37	
B	11	5	0.0095*
C	11	5	
Peripheral blood lymphocytosis (/μL)	44,383 ± 7[illegible]	1[illegible]60 ± 9,613	0.0015°
Pattern of bone marrow infil[illegible]			
Nodular/interstitial	[illegible]	21	0.0006*
Diffuse	[illegible]	3	
Surface Ig de[illegible]			
Low	43	46	ns*
Higi	1	2	
FMC7 e[illegible]sion			
Positive	35	35	ns*
Negative	9	13	
CD79b expression			
Positive	38	35	ns*
Negative	6	13	
CD38 expression			
Positive	31	34	ns*
Negative	13	14	
CD20 ABC values	10,022±9,137	12,231±11,214	ns°
CD22 ABC values	7,639±7,001	6,232±4,210	ns°
Cytogenetics#			
Trisomy 12	0	9	0.03*
Del 13q14	7	1	

*Data are expressed as number of cases displaying or not CD69 molecule with the exception of peripheral blood lymphocytosis, CD20 and CD22 ABC values, reported as mean±standard deviations. °Mann-Whitney test; *Chi-squared test; §evaluated only in 43 cases; #evaluated only in 27 cases.*

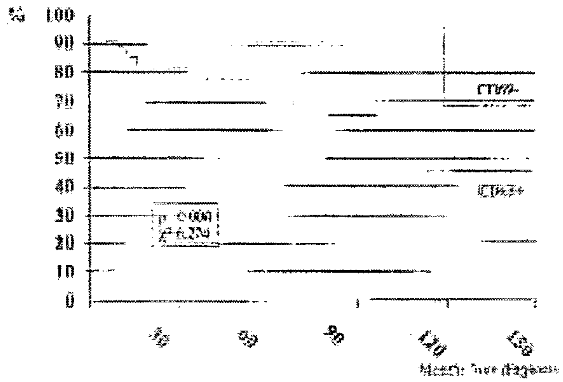

Figure 1. Actuarial survival based on CD69 expression in B-CLL (Kaplan-Meier and Pearson's correlation test).

Thus in our hands, CD69 was found to be expressed on neoplastic B-cells of more than half of our B-CLL patients. Since this expression correlated with worse clinico-biological findings as well as a shorter survival than did CD69-negative forms, CD69 could be considered as a new promising immunologic prognostic parameter in B-CLL. However, the exact role of CD69 in th pathogenesis and clinical behavior of B-CLL remains to be b ter established, needing further investigations.

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ROLE OF CYTOKINES IN RHEUMATOID ARTHRITIS

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KEY WORDS: rheumatoid arthritis, tumor necrosis factor, interleukin 1, interleukin 10

Abstract

Analysis of cytokine mRNA and protein in rheumatoid arthritis tissue revealed that many proinflammatory cytokines such as TNFα, IL-1, IL-6, GM-CSF, and chemokines such as IL-8 are abundant in all patients regardless of therapy. This is compensated to some degree by the increased production of anti-inflammatory cytokines such as IL-10 and TGFβ and cytokine inhibitors such as IL-1ra and soluble TNF-R. However, this upregulation in homeostatic regulatory mechanisms is not sufficient as these are unable to neutralize all the TNFα and IL-1 produced.

In rheumatoid joint cell cultures that spontaneously produce IL-1, TNFα was the major dominant regulator of IL-1. Subsequently, other proinflammatory cytokines were also inhibited if TNFα was neutralized, leading to the new concept that the proinflammatory cytokines were linked in a network with TNFα at its apex. This led to the hypothesis that TNFα was of major importance in rheumatoid arthritis and was a therapeutic target. This hypothesis has been successfully tested in animal models, of, for example, collagen-induced arthritis, and these studies have provided the rationale for clinical trials of anti-TNFα therapy in patients with long-standing rheumatoid arthritis. Several clinical trials using a chimeric anti-TNFα antibody have shown marked clinical benefit, verifying the hypothesis that TNFα is of major importance in rheumatoid arthritis. Retreatment studies have also shown benefit in repeated relapses, indicating that the disease remains TNFα dependent. Overall these studies demonstrate that analysis of cytokine expression and regulation may yield effective therapeutic targets in inflammatory disease.

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INTRODUCTION

Cytokines are local protein mediators, now known to be involved in almost all important biological processes, including cell growth and activation, inflammation, immunity, and differentiation. Thus, it is not surprising that they have a role in an autoimmune disease such as rheumatoid arthritis (RA), in which there is chronic inflammation, with fibrosis and the eventual destruction of cartilage and bone.

As all the cytokines cloned first, such as interferon gamma (IFNγ), interleukin 2 (IL-2), tumor necrosis factor (TNFα), and interleukin 1 (IL-1), were mediators of immunity or of proinflammatory activity, the underlying assumption in the initial investigations into the role of cytokines in RA was that those most abundantly expressed were likely to be pathogenic. Thus, the long-term goal of cytokine analysis in RA was to define new targets for therapy. It is now clear that this early view of the role of cytokines in arthritis was far too simple. We now know that many cytokines with proinflammatory features also have anti-inflammatory aspects (e.g. IFNγ), and that those with mostly anti-immune/inflammatory features, such as transforming growth factor β (TGFβ) or interleukin 10 (IL-10), also have proinflammatory effects and hence are potentially pathogenic.

In this review, we focus on RA because it is the most studied form of arthritis, and we compare it with other forms of arthritis where possible, including animal models. Discussion of the role of cytokines in other autoimmune diseases can be found elsewhere, including books recently published (1, 2). The analysis of cytokine expression and regulation in RA, as described here, identified a therapeutic target TNFα, which led to the successful clinical trials using a monoclonal antibody to TNFα. These in vivo studies have confirmed the usefulness of the in vitro analysis performed on RA synovial membrane cell cultures.

THE PATHOGENESIS OF RHEUMATOID ARTHRITIS

Although the etiology of RA remains elusive, susceptibility factors are evident. Thus, the threefold predominance of RA in women may be attributable to hormonal factors, and the clear-cut genetic contribution in this disease is contained predominantly within the HLA class II locus. On one side of the peptide binding groove of HLA-DR, there is a shared epitope, comprising amino-acids 70-74 of the β chain, which is conserved in the DR1 and DR4 disease-susceptible haplotypes (3). This epitope is present in > 80% of Caucasian RA patients and is the most important evidence to support the concept that T lymphocyte recognition is important at some stage in the pathogenesis of RA, either in shaping the T

cell receptor (TCR) repertoire or in the presentation of an inducing microbial or autoantigenic peptide.

More recently, other candidate genes including cytokine polymorphisms have been investigated. Although an allele polymorphism in the promoter region of TNFα associated with HLA A1, B8, and DR3 was identified and another in the promoter region of the IL-1α gene, these are *not* associated with RA but are with other autoimmune diseases such as systemic lupus erythematosus (SLE) in the case of the TNFα polymorphism (4, 5) and a severe form of arthritis in children (JRA) in the case of IL-1α (6). The discordance in developing rheumatoid disease between identical twins (7) clearly suggests that nongenetic factors are also important, and infectious agents are the most plausible explanation. However, although many infectious agents have been implicated over the years, from viruses to mycoplasma and mycobacteria, none have yet been reproducibly identified in different laboratories.

The pathology of RA extends throughout the synovial joint (Figure 1), and in severe cases involves many other organs. In contrast to the acellular nature of normal synovial fluid, RA synovial fluid is enriched predominantly with neutrophils, but macrophages, T lymphocytes, and dendritic cells are also present. The increase in cellularity, however, is most obvious in the synovial membrane, which becomes infiltrated by cells recruited from the blood. The lining layer of the joint is increased, from 1–2 cells to 6–8 cells thick, and is comprised mostly of activated macrophages (occasionally referred to as *type-A* synoviocytes) with an underlying layer of fibroblast-like cells (occasionally referred to as *type-B* synoviocytes). The deeper layers within the synovium have follicles of lymphoid cells around vessels as well as lymphocytes scattered between them. Neovascularization is prominent, and there are many activated endothelial cells. The most abundant cells in the synovial membrane are macrophages and T lymphocytes, but plasma cells, dendritic cells, and activated fibroblasts are also found. Many of these cells are activated and express abundant HLA class II and adhesion molecules of relevance in antigen presentation (8-13).

The major site of irreversible tissue damage originates at the junction of the synovium lining the joint capsule with the cartilage and bone, a region often termed the *pannus*, an area rich in macrophages. The cells of the pannus migrate over the underlying cartilage and into the subchondral bone, causing the subsequent erosion of these tissues (14). The destruction of the cartilage seen in rheumatic disease is now considered to be mostly due to the activity of matrix metalloproteinases (MMPs), enzymes produced by activated macrophages and fibroblasts in response to proinflammatory cytokines such as IL-1 and TNFα. These enzymes are synthesized and secreted as latent molecules, with

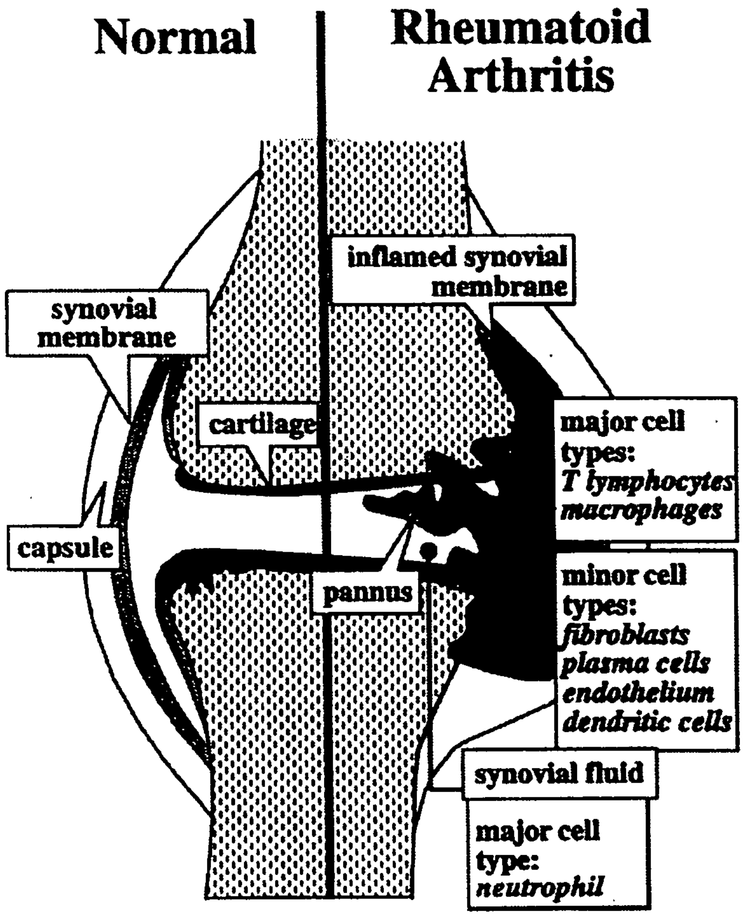

Figure 1 Diagrammatic representation of synovial joint, normal joint (*left*) rheumatoid arthritis joint (*right*).

activation brought about by proteolytic cleavage of the propeptide domain. In arthritis, the MMP enzymes collagenase (MMP-1) and stromelysin 1 (MMP-3), whose production is increased, play an important role in the destructive process (15). The activity of MMPs is regulated to some extent by tissue inhibitors of metalloproteinase (TIMP), three forms of which have been cloned in humans; these irreversibly bind the active MMP to form a 1:1 complex with the enzyme. The fact that the TIMPs are produced by the same cells that produce the MMPs suggests an intimate role for these inhibitors in regulating matrix turnover, and also that much of the connective tissue destruction associated with arthritic disease is due to an imbalance between the production of the MMPs and that of the specific TIMPs. Of interest in this regard is the observation that transforming growth factor β (TGFβ) and IL-10, two immunoregulatory and anti-inflammatory cytokines produced in the RA joint (discussed in the

next section), not only inhibit the production of proinflammatory cytokines that induce MMPs, but also induce the production of their native inhibitors TIMPs (16) (J-M Dayer, personal communication).

In addition to the MMPs, other enzymes synthesized by cells resident within the cartilage and bone as well as by infiltrating inflammatory cells are capable of cleaving the major components of cartilage and bone such as proteoglycan and collagen (type I, II, IX, X, and XI). These enzymes include serine, aspartic, and particularly cysteine endopeptidases such as cathepsin B (17). Detailed discussion of these enzyme effects are beyond the scope of this chapter.

CYTOKINE EXPRESSION IN RHEUMATOID ARTHRITIS

Although the major pathology in RA occurs in the synovium, synovial fluid is more readily accessible, and as such the production of cytokines such as IL-1 was first documented in this compartment (18). However, the relevance of cytokines found in synovial fluid in the pathogenesis of the disease is unclear. This fluid is largely a sink containing a complex mixture of molecules, including a large concentration of hyaluronan, other proteoglycans, degradative enzymes, and serum proteins, many of which inhibit or degrade cytokine function. Furthermore, as the pathways and factors controlling the flux into the joint space are ill understood, we have not pursued analysis of cytokines in synovial fluid. In contrast, cytokine expression in synovial membrane is likely to be of greater relevance to the pathogenesis of RA, as this is the principal site of immune and inflammatory activity. The source of human RA synovial membrane is usually joint replacement surgery, which can provide large numbers of cells but has the limitation that this tissue is obtained only from late stages of the disease. On occasion, small, active samples from early stage disease are available from arthroscopic biopsies.

Since cytokines are local messenger molecules, we first investigated mRNA production as an index of synthesis. Due to the restricted sample size available and the large number of potentially relevant mediators to probe for, Northern blot hybridization and slot blotting techniques were developed for this purpose. With the capacity to strip and reprobe filters a number of times, it became possible to rapidly generate abundant data on cytokine expression in the rheumatoid synovium (19, 20). These techniques have now been replaced by more sensitive methods such as reverse transcriptase polymerase chain reaction (RT-PCR).

Pro-Inflammatory Cytokines

IL-1 and TNFα protein were readily detected in synovial fluid (18, 21–24). In the synovium, at the mRNA level these cytokines can be detected by blotting

(20) and by in situ hybridization (25, 26). Immunohistological localization of these protein products has demonstrated predominant expression in macrophages (25, 27). These proteins were also detected in the short term in in vitro cultures of the entire mixture of cells derived by enzymatic disaggregation of the synovial membrane (20, 28). Of importance was the observation that IL-1 and TNFα could be detected by bioassay of synovial membrane cultures, and hence they were present in quantities able to signal effectively.

Subsequently, as other proinflammatory cytokine and growth factor cDNAs were cloned, their mRNAs and proteins were also detected in RA synovial tissue. These cytokines included interleukin 6 (IL-6) (29–32), interferon α (IFNα) (24), granulocyte macrophage colony stimulating factor (GM-CSF) (33–35), macrophage colony stimulating factor (M-CSF) (36), and leukocyte inhibitory factor (LIF) (37–39). Many of these studies used osteoarthritis tissue or synovial fluid for comparison. Usually the same cytokines were produced, but at a lower level.

Currently we are unaware of any published data concerning the proinflammatory cytokine interleukin 12 (IL-12) (40) in rheumatoid joints or cultures. In our laboratory (FM Brennan, H Thomssen, P Green, RN Maini, M Feldmann, unpublished data), we have detected low levels of IL-12 in supernatants from RA joint cell cultures. The levels were within the biologically active range (20–200 pg/ml), with a mean of 79 pg/ml for p40 IL-12, and a mean of 30 pg/ml for p75 heterodimer. It is thus conceivable that IL-12 is important in the late stages of the disease process. In view of the predominance of CD4$^+$ Th1 cells in RA joints, with very few Th2 cells (see later), and that IL-12 is a powerful stimulus for skewing the Th1/Th2 ratio (41), it is likely that early in the disease process IL-12 may be importantly involved. In the latter stages IL-12 may also be involved in maintaining the Th1 preponderance and in driving cytokine production. Neutralizing experiments are needed to evaluate its potential role in the latter process. In collagen-induced arthritis, it has recently been reported (42) that IL-12 may replace the need for mycobacteria, markedly augmenting the incidence of arthritis in DBA/1 mice injected with Freund's incomplete adjuvant and collagen type II.

The cytokines identified as major contributors in the hyperplasia of rheumatoid synovial fibroblasts include platelet-derived growth factor (PDGF) (43–45), fibroblast growth factor (FGF), (45–47), and transforming growth factor β (TGFβ) (45, 47–50). Which of these is of major importance has not been evaluated experimentally, although immunohistological techniques have demonstrated co-expression of PDGF and FGF (45, 51) and of basic FGF with TGFβ (47) in RA synovial tissue. The presence of these and other cytokines in RA synovium is summarized in Table 1.

Table 1 Cytokine expression in RA Synovial Tissue

Cytokine		mRNA	Protein	References
Proinflammatory Cytokines				
IL-1α & β	(interleukin 1)	Yes	Yes	(18, 20, 23, 25, 26 28, 93, 103, 116)
TNFα	(tumour necrosis factor alpha)	Yes	Yes	(21–28)
LT	(lymphotoxin)	Yes	+/−	(22, 96)
GM-CSF	(granulocyte macrophage colony stimulating factor)	Yes	Yes	(33–35)
M-CSF	(macrophage colony stimulating factor)	Yes	Yes	(36)
IL-6	(interleukin 6)	Yes	Yes	(25, 29–32)
LIF	(leucocyte inhibitory factor)	Yes	Yes	(37, 39)
IL-II	(interleukin II)	?	?	
Onco M	(Oncostatin M)	?	?	
IL-2	(interleukin 2)	Yes	+/−	(19, 36)
IL-3	(interleukin 3)	No	No	(36)
IL-7	(interleukin 7)	?	?	
IL-7	(interleukin 9)	?	?	
IL-15	(interleukin 15)	Yes	Yes	(162)
IFNα	(interferon alpha)	Yes	Yes	(24)
IFNβ	(interferon beta)	?	?	
IFNγ	(interferon gamma)	Yes	+/−	(19, 25, 96)
IL-12	(interleukin 12)	Yes	Yes	(unpublished Feldmann M)
Immunoregulatory Cytokines				
IL-4	(interleukin 4)	?	No	(84)
IL-10	(interleukin 10)	Yes	Yes	(67, 85, 91, 92)
IL-13	(interleukin 13)	Yes	Yes	(unpublished Klareskog, L)
TGFβ	(transforming growth factor beta)	Yes	Yes	(48, 48, 50, 68, 68–71, 84)
Chemotactic Cytokines				
IL-8	(interleukin 8)	Yes	Yes	(53–55, 60, 61)
Gro α	(melanoma growth stimulating activity)	Yes	Yes	(60)
MIP-1α	(macrophage inflammatory protein 1 alpha)	Yes	Yes	(60, 63)
MIP-1β	(macrophage inflammatory protein 1 beta)	Yes	Yes	(60)
MCP-1	(monocyte chemoattractant protein 1)	Yes	Yes	(56–60)
ENA-78	(epithelial neutrophil activating peptide 78)	Yes	Yes	(62)
RANTES	(regulated upon activation T cell expressed & secreted)	Yes	Yes	(64)
Mitogenic Cytokines				
BDGF	(Vascular endophelial cell growth factor)	Yes	Yes	(184, 185)
FGF	(Fibroblast growth factor)	Yes	Yes	(44, 46, 47, 50, 51)
PDGF	(Platelet-derived growth factor)	Yes	Yes	(43, 45, 51)

Chemotactic Cytokines (Chemokines)

Many of the features of the rheumatoid synovial environment, such as the selective accumulation of VLA4$^+$ CD45RO$^+$ T cells and activated macrophages in the membrane, and of polymorphonuclear cells in joint fluids, suggest a possible rôle for leukocyte chemoattractant molecules such as chemokines. This superfamily of low MW peptides (7–15 kDa) has a conserved four-cysteine motif and consists of at least two subfamilies: the C-X-C (α) chemokines such as interleukin 8 (IL-8), melanoma growth stimulating activity (GRO α), and epithelial neutrophil activating peptide 78 (ENA 78), all of which predominantly attract neutrophils; and the C-C (β) chemokines such as RANTES (regulated upon activation normal T cell expressed and secreted), MCP-1 (monocyte chemoattractant protein 1), and MIP-1α (macrophage inflammatory protein 1α), which chiefly recruit T cells and monocytes (52).

Chemokines could be released by a number of cells present in RA joints, including endothelial cells, fibroblasts, macrophages, and lymphocytes. Members of both subclasses have been implicated in the pathogenesis of RA. Data from our laboratory and others have demonstrated the presence of IL-8 in RA synovial membrane cells (53–55); the production of other chemokines including GROα, MIP-1α, macrophage inflammatory protein 1β (MIP-1β) and MCP-1 (56–60) has subsequently been reported. In addition, immunohistochemical analyses of RA synovial tissue have demonstrated the presence of IL-8 (61), ENA-78 (62), MIP-1α (63), MCP-1 (57), and RANTES (64), predominantly associated with synovial tissue macrophages and, to a lesser extent, with endothelium and synovial tissue fibroblasts. Because the majority of the cells in the RA synovium are macrophages and T lymphocytes, β chemokines are likely to be important, although neutrophil chemoattractants such as IL-8, GROα, and ENA-78 may play a role in neutrophil accumulation within the synovial fluid.

Finally, in addition to their leukocyte chemotactic activity, chemokines may also be involved in other processes relevant to RA, such as connective tissue metabolism (for example, the α-chemokine connective tissue activation peptide CTAP III is elevated in rheumatoid plasma) and neovascularization (IL-8, GROα, ENA-78). However, it has not yet been ascertained which one(s) are the major contributors to leukocyte infiltration, or which will be therapeutically relevant. More recently, Kunkel and coworkers demonstrated expression of the β-chemokines MCP-1 and MIP-1α, and the α-chemokines ENA-78 and MIP-2 (the murine functional homolog of IL-8) (65) in murine collagen-induced arthritis (CIA). The earliest detectable levels of MIP-1α, MCP-1 and MIP-2 were observed 4 weeks after initial collagen challenge, a time-course paralleling that of disease development, whereas the time-course for ENA-78 was much slower

(8 weeks). Passive immunization of CIA mice with antibodies against either MIP-1α or MIP-2 resulted in both a delay in disease onset and a decrease in disease severity. Interestingly, anti-IL-10 treatment increased the expression of MIP-1α and MIP-2, associated with enhanced leukocyte infiltration in the joints, suggesting a homeostatic role for IL-10 in chemokine regulation (66) as well as its previously reported role (67) in regulating TNFα and IL-1β.

INHIBITORS OF IMMUNE ACTIVATION AND INFLAMMATION

Cytokines generally regarded as possessing immunoregulatory and inhibitory properties were discovered and subsequently cloned after the proinflammatory cytokines. These molecules include TGFβ, IL-4, IL-10, and IL-13. A number of studies from different groups have documented that TGFβ is abundant in both the precursor, inactive, form and the active form in rheumatoid joints (48, 68–71). However, whether TGFβ actually functions in RA as an anti-inflammatory cytokine has been questioned because it also has the potential to be proinflammatory (72). Thus, if injected locally into the joints of normal rats, TGFβ resulted in a rapid leukocyte infiltration with synovial hyperplasia leading to synovitis (73, 74), whereas if injected systemically into rodents susceptible to arthritis, it antagonized the development of polyarthritis (75, 76). Furthermore, in a recent publication, it was reported that anti-TGFβ antibody, injected locally into the joint of rats with arthritis, diminished the ongoing inflammation (77). These studies indicate the multipotential properties of TGFβ, and the differential effects if injected systemically or locally into the joint. Moreover, TGFβ clearly has other proinflammatory effects, such as acting as a chemotactic factor for monocytes (78). On the other hand TGFβ is likely to be a key cytokine involved in repair and fibrosis in the joints. For example, while inhibiting production of metalloproteinases such as collagenase (68) and inducing TIMP (16), TGFβ also stimulates the production of type I and type XI collagen (79). Thus, locally TGFβ may promote reparative processes in arthritic synovial connective tissue scarring and tissue repair by inhibiting cartilage and bone destruction. However, in chronic lesions, overproduction of TGFβ could contribute to the ongoing damage by recruiting inflammatory macrophages and fibroblasts with the potential for tissue destruction, and also, as shown recently (RA Fava, unpublished observation), by its ability to promote angiogenesis through induction of vascular endothelial cell growth factor (VEGF).

In common with TGFβ, IL-4 also displays some immunoregulatory effects such as inhibition of LPS-induced IL-1, TNFα, PGE2, and 92-kDa gelatinase

production in human monocytes (80–83). However, in contrast to TGFβ, IL-4 has not been found in rheumatoid synovial tissue cultures (84) nor in T cells cloned from RA synovial biopsies (85), although it has been detected in reactive arthritis (86). This and other evidence suggest that CD4$^+$ve Th2-derived cytokines are not abundant in RA joints, and that CD4$^+$ve Th1 cells predominate in this site (87). It is possible that the lack of IL-4–producing CD4$^+$ Th2 cells contributes to the pathogenesis of RA, and this has led to suggestions that IL-4 may be a useful therapeutic agent (88) (NA Mitchison, unpublished observation). This latter group demonstrated that the addition of recombinant IL-4 to RA synovial tissue organ cultures resulted in the inhibition of proinflammatory cytokine production (88). Using dissociated synovial cell cultures (89), we did not observe such a significant inhibition of proinflammatory cytokine production with IL-4; indeed, the expression of TNF-R on the surface of cells increased significantly, which could conceivably increase the responsiveness to TNFα. These effects aside, it is interesting to note that IL-4 production in RA joints is defective, and there is a report suggesting that the incidence of allergies is lower in RA patients (90).

IL-10 also has profound anti-inflammatory and immunoregulatory effects. Its presence has been documented in RA peripheral blood (91) and synovial joints by RT-PCR of fresh frozen biopsies, immunostaining of fresh frozen biopsies, and by assay of 24-h culture supernatants of dissociated joint cell cultures (67, 92). Furthermore, we found that in these RA synovial cell cultures, the endogenous IL-10 produced is functional, since inhibition of its activity using a neutralizing monoclonal antibody enhanced TNFα and IL-1 production (67). Conversely, addition of recombinant IL-10 to these cultures inhibited TNFα and IL-1 production by approximately 50%. In a similar study but using synovial tissue organ cultures (93), exogenous IL-10 also inhibited IL1β although IL-4 was more potent, and additionally IL-4 (but not IL-10) induced the production of the native inhibitor of IL-1, the IL-1 receptor antagonist, (IL-1ra). Although IL-10 is not a potent inducer of IL-1ra, we have shown recently that IL-10 (but not IL-4) induces the production of the endogenous TNF inhibitors, i.e. soluble TNF receptors from monocyte cultures, while also downregulating surface TNF receptor (TNF-R) expression (94). Thus, many of the properties of IL-10 are compatible with its being a major immunoregulator. However, not all of its properties are immunosuppressive, and its B cell stimulatory effects (95) may be important in the production of rheumatoid factors.

IL-13 also has inhibitory features resembling those of IL-4. It has not yet been quantitated in RA joints. However, preliminary data (L Klareskog, unpublished observation) suggest it can be detected by immunostaining in RA synovium.

ARE THERE DISCREPANCIES BETWEEN MRNA AND PROTEIN LEVELS FOR CYTOKINES IN RA JOINTS?

In contrast to the cytokines discussed in the previous section (IL-1, TNFα, IL-6, IL-8, GM-CSF), other cytokines detectable at the mRNA level were not abundant at the protein level but were present at low levels. These include cytokines derived principally from T cells, including IL-2, lymphotoxin (LT), and IFNγ (19, 36, 96). However, more recently, using more sensitive methods, T cell–derived cytokine proteins have been visualized by immunostaining; with this procedure, several groups have shown that IFNγ-producing T cells are not rare, with up to 0.5% of the total synovial T cells expressing IFNγ at any one time in joints of longstanding RA patients (85, 97) (L Klareskog, JS Smolen, unpublished observation). Other T cell cytokines, however, were not often detectable at either protein or mRNA level, e.g. IL-4 (84).

A variety of conclusions have been drawn on the basis of these observations. The extreme position has been taken by Firestein & Zvaifler (98), who have used these results as an argument that T cells do not have much role in the chronic, established stages of rheumatoid arthritis; instead, they argue, the process is perpetuated by cytokine interactions between other cells, including fibroblasts and macrophages. Alternative interpretations of the same data are possible, in view of the different physiology of T cells and macrophages. First, the low frequency of Th1-secreted products such as IFNγ and IL-2 may be sufficient to maintain T cell–dependent immune inflammation. Second, T cells migrate to their target tissue and deliver cytokines in a polarized manner, directly to their target cell. It would thus not be anticipated that large amounts of cytokines should be present in supernatants of T cells. Thirdly, since the evidence that T cells produce only low levels of cytokines, it has become apparent that cytokine inhibitors are abundant in body fluids. For IL-2, IFNγ, LT, etc, these are the soluble cytokine receptors, shed from the surface of cells. These effects would all contribute to limited detection of T cell cytokine. The major cause of limited T cell cytokine detection now appears to be that chronic inflammatory sites such as RA joints contain abundant quantities of cytokines capable of diminishing T cell cytokine synthesis. These include TGFβ (99), but probably the most important in RA is IL-10 (100, 101), since the addition of a neutralizing anti-IL-10 antibody to RA synovial membrane culture was able to upregulate IFNγ production in a proportion of cultures within 24 h (67).

CYTOKINE REGULATION

From the beginning of studies of cytokine expression in RA synovium, aspects of cytokine regulation there appeared different from what may have been

expected for in vitro activated cells. Most important was the consistent pattern of cytokine production, with all samples producing essentially the same pattern of cytokines. This was regardless of the duration of the disease or therapy, even with potential cytokine synthesis inhibitory drugs such as corticosteroids. In stimulated macrophages, IL-1β production greatly exceeds that of IL-1α at mRNA or protein level (102). In RA joints the production of IL-1α mRNA was relatively high, suggesting that IL-1 was either regulated differently or mostly was not coming from activated cells of the macrophage lineage (20).

The regular presence of cytokines in all rheumatoid joint synovial samples suggested that, unlike what is reported with normal cells stimulated in vitro, where cytokine expression is transient, cytokine expression in RA synovial tissue was likely to be prolonged or even continuous. This hypothesis was evaluated by placing RA synovial membrane cells in culture in the absence of extrinsic stimulation and ascertaining mRNA levels at various times. For example, in RA cultures, IL-1α mRNA persisted for the duration of the culture period (5–6 days) in the absence of extrinsic stimulation; in contrast in mitogen-stimulated peripheral mononuclear points cell cultures, mRNA expression was transient and shut off within 24 h (20).

The above results indicated that the signals regulating prolonged cytokine synthesis in rheumatoid joint cells were present in these RA synovial membrane cell cultures and hence could be analyzed. The problem at face value was quite daunting, with a heterogeneous mixture of cells producing a plethora of cytokine and noncytokine signals. Since Fell, Saklatvala and others (103–105) had shown that IL-1 (described as 'catabolin') was of importance in the initiation of destruction of cartilage and bone, we chose initially to analyze the regulation of IL-1 in rheumatoid synovial membrane cell cultures. While LPS is the usual experimental inducer of IL-1, possibly relevant in reactive arthritis, it is not likely to be important in RA: Instead we evaluated the effect of blocking cytokine inducers of IL-1. Since TNFα reportedly was a potent inducer of IL-1 (106), we used neutralizing antibodies to TNFα and lymphotoxin in RA synovial cultures to evaluate its role. The results were striking: within 3 days, IL-1 bioactivity had virtually disappeared. IL-1 mRNA levels were reduced much earlier. From this experiment, we concluded that TNFα was the major signal driving IL-1 synthesis in RA SM cultures (28).

This result prompted analysis of the production of other cytokines dependent on TNFα. Anti-TNFα antibodies also inhibited the production of another proinflammatory cytokine, GM-CSF (34). In addition to being a growth factor for monocytes in hematopoiesis, GM-CSF also activates mature monocytes and macrophages. It has been implicated in the pathogenesis of RA, based on the observation that it is produced spontaneously in RA synovial cell cultures.

GM-CSF induces and maintains HLA class II expression on RA synovial cells (35), in addition to regulating myelopoiesis (107). It may also affect other cell types in RA tissue as it can augment neutrophil-mediated cartilage degradation and adherence (108). In addition to GM-CSF, other cytokines including IL-6, IL-8 (109), and IL-10 (67) were all found to be induced by TNFα in these RA synovial cell cultures. The results obtained with pathological human tissue (synovium) are probably a reflection of the events that take place in a normal host defense response, as judged by the sequential appearance of TNFα, IL-1, and IL-6 in the serum of mice or monkeys injected with LPS or gram negative bacteria, and on the greatly reduced IL-1 and IL-6 production that follows neutralization of TNFα by an anti-TNFα antibody (110).

The role of TNFα in upregulating the production of other proinflammatory cytokines is not a completely unidirectional process. It has been reported that TNFα production can be upregulated by IL-1, GM-CSF, and IFNγ, and it is likely that such effects do take place in vivo. However the cytokine interactions are not symmetrical. Thus, blocking IL-1 in RA joint cell cultures using recombinant IL-1 receptor antagonist (IL-1ra) protein does not diminish TNFα production, but it downregulates IL-6 and IL-8 production (109). There is evidence that proinflammatory cytokines such as TNFα or IL-1 have an autocrine effect and can regulate their own synthesis. However, little evidence suggests that this is important in the RA joint: IL-1ra does not inhibit the production of IL-1α or IL-1β as judged by ELISA (109).

TNFα seems to be the cytokine at the apex of the proinflammatory cytokine cascade or network. So an important question is: What regulates TNFα production in rheumatoid joints? Currently this is unresolved. However, we have preliminary evidence that it is T cell–dependent, as T cell depletion in RA joint cell cultures diminishes TNFα production (FM Brennan, C Hawrylowicz, RN Maini, M Feldmann, unpublished observation). However, the critical T cell–derived signals to the monocytic cells, the major source of TNFα, are not yet known. Dayer and his colleagues (111, 112) have produced evidence that cell surface signals from T cells are involved in regulating IL-1 and probably TNFα in co-cultures of T cells and macrophages, with CD69 and CD11β playing an important role in this process.

CYTOKINE RECEPTORS

While it is useful to document the full range of cytokine expression in an inflammatory site, in isolation this information is not necessarily indicative of the extent or potential for cytokine signaling. The final outcome ultimately depends on two other important features, namely, the appropriate expression of cytokine receptors and the local concentration of cytokine inhibitors.

With the identification of cytokine receptors, the cDNA cloning and expression of many receptor chains, and the production of relevant antibodies, it has become possible to study cytokine receptor expression in small samples of diseased tissue. The technology used for receptor analysis has changed from measurement of mRNA expression, initially used for the IL-2Rα (or Tac) chain (113), to radioligand binding through to the current use of monoclonal antibodies. With the realization that most cytokine inhibitors are derived from the surface receptor by enzymatic receptor cleavage, it is evident now that mRNA analysis for cytokine receptors is not informative, as it does not discriminate between the cell surface signaling form and the inhibitory soluble forms.

TNF receptor expression has been studied most thoroughly. Both the p55 and p75 TNF surface receptors are upregulated in active RA tissues at both the protein and mRNA level (114). This is the case all over the synovium, including the areas abutting the sites of erosion (115) and in the endothelial cells. Of particular interest was the observation that macrophages at the cartilage pannus junction and the endothelial cells also produce TNFα (Figure 2), indicating the potential for autocrine stimulation. The expression of other cytokine receptors for IL-1 (116), GM-CSF (117), and IL-6 (unpublished) has been analyzed, but whether levels are normal or altered is not clear.

SOLUBLE CYTOKINE RECEPTORS

It is now well documented that the extracellular ligand-binding domain of most of the single transmembrane cytokine receptors is also found in biological fluids in a soluble form. Most of these soluble receptors are cleaved from the surface receptors of the cells by proteolytic enzymes, as in only a few instances is there evidence for a truncated mRNA, generated by alternative splicing (118, 119).

The first soluble receptor to be convincingly documented was that derived from the IL2Rα chain (113), but as this is of low affinity, its functional relevance as a cytokine inhibitor in vivo has not been clearly documented. TNF inhibitors were detected in urine and serum almost concurrently in 1988–1989, by the groups of Dayer (120), Olsson, (121) and Wallach (122), and they were subsequently characterized as the extracellular domains of the two TNF receptors. Using antibodies to these proteins, ELISA assays were developed, and soluble TNF-R (sTNF-R) were observed in normal serum at levels of 1–4 ng/ml, with the p55 lower than p75. In RA serum, levels of both were elevated, and even more so in the synovial fluid, where levels 3–4 times those of serum are reached. Furthermore, sTNF-R levels in plasma correlated with disease activity (123, 124). These results of upregulated TNF inhibitor in RA both systemically and locally are of interest, as they help exclude the possibility that a major contribution to the pathogenesis of RA is failure to produce inhibitory

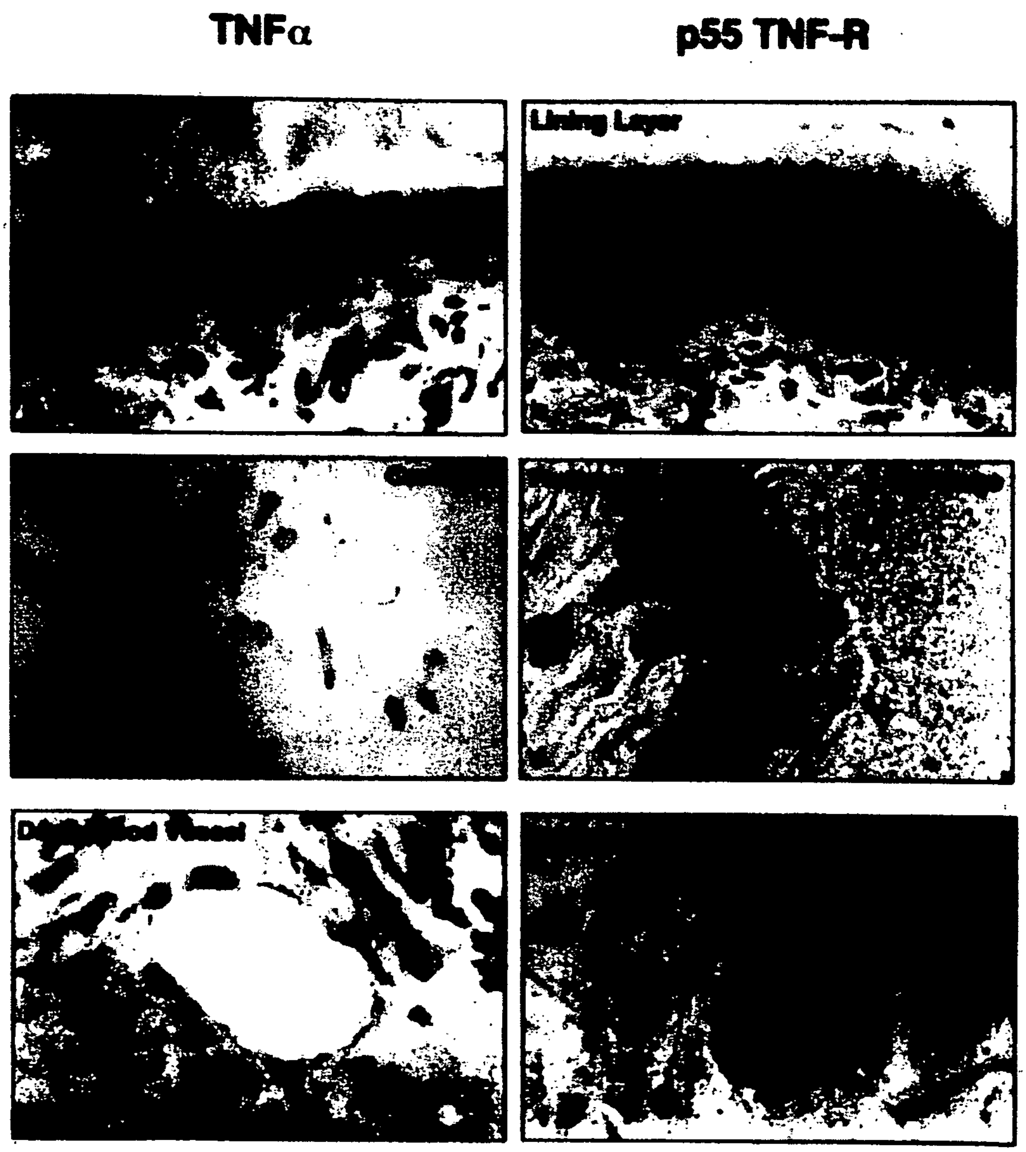

Figure 2 TNFα and p55 TNF-R expression in rheumatoid synovium. TNFα (*left panel*) and p55 TNF-R (*right panel*) colocalization was demonstrated by immunohistology in synovial lining layer (*a* and *b*) at the cartilage pannus junction (*c* and *d*) and in endothelial cells in deep blood vessels (*e* and *f*). Modified and reprinted by kind permission of *Arthritis Rheum.* (Chu et al, 1989, 34:1125–32 and Deleuran et al 1992, 35:1170–78).

factors normally. Indeed we observed that in RA synovial cultures sTNF-Rs were produced at concentrations capable of neutralizing a significant proportion of the TNFα generated (125). These results indicate that local production of cytokine inhibitors is capable of diminishing disease activity, and cytokine activity is partially downregulated by endogenous inhibitors.

Soluble IL-1 receptor has also been detected in RA tissues, initially in synovial fluid (126). This was first found as an IL-1β binding protein and was subsequently identified using monoclonal antibody as the type II IL-1R. This receptor is not involved in signaling and appears to function not only as a decoy on the cell surface, but also as an inhibitor (in its soluble form) as it binds proIL-1β (preventing its processing) and mature IL-1β, but not IL-1ra (127). Other soluble cytokine receptors have also been identified in RA. These include soluble IL-6 receptor (FM Brennan, A Cope, P Green, RN Maini, D Novick, M Feldmann, unpublished observation), which is not an antagonist but, in contrast, is an agonist (128, 129), and soluble IFNγ receptors (FM Brennan, A Cope, P Green, RN Maini, D Novick, M Feldmann, unpublished observation).

IL-1 Receptor Antagonist

The third member of the IL-1 family, the IL-1 receptor antagonist (IL-1ra) is the only known cytokine receptor antagonist (130). It has high affinity for membrane IL-1 receptor (type I and II), but due to the capacity of IL-1 to activate cells at very low receptor occupancy, a considerable molar excess ($\approx 100:1$) of IL-1ra is needed to inhibit IL-1. The expression of IL-1ra has been analyzed in RA joints; thus mRNA levels are upregulated, as is production of the protein in RA synovial fluid and joint cell cultures (131–134), which is immunolocalized to CD68 positive macrophages within the synovium (116, 132). Of key importance is the ratio of IL-1ra to IL-1, which in a recent report (135) from RA synovial cultures ranged from 1.2 to 3.6, well below the 100-fold excess of IL-1ra needed to neutralize IL-1 bioactivity. Thus, it is not surprising that bioactive IL-1 is found in the majority of RA culture supernatants (28). Normal joint tissues express very little IL-1ra, so IL-1ra production is upregulated in the disease process but not sufficiently to neutralize IL-1. It is of interest that a study of the ratio of IL-1ra:IL-1 in the synovial fluid of patients with Lyme arthritis indicated that the patients with the most favorable outcome had the highest ratio (136).

CYTOKINE ANALYSIS IN ANIMAL MODELS

While there is no animal model of arthritis that entirely mimics RA, a variety of models have been generated that resemble RA in many important respects. These models are being analyzed to provide possible insights into the

pathogenesis of RA and to help develop therapeutic strategies. There is very little published data concerning cytokine expression in the synovium in animal models. Most of the available data concerning the role of cytokines in animal models have emerged from administering cytokines into the joints of mice or from therapeutic studies in these mice. More recently the development of transgenic mouse models of arthritis has provided an alternative way in which to analyze the role of cytokines in arthritis.

Originally a synovitis with proteoglycan degradation was seen to occur if IL-1 alone, or in combination with TNFα, was injected into the joint of a rabbit (137, 138). Subsequently, injection of IL-1 (139, 140) or TNFα (141, 142) into collagen-immunized mice or rats accelerated the onset and increased the severity of arthritis. Therapeutic studies in mice indicated the involvement of these proinflammatory cytokines in the normal course of arthritis. For example, several groups have shown that collagen-induced arthritis (CIA) in mice may be treated effectively with anti-TNF antibody or other TNFα inhibitors. Thus, monoclonal antibodies to TNF ameliorated CIA when administered prior to disease onset (143, 144). In addition, anti-TNF monoclonal antibody treatment was used successfully after disease onset and was found to reduce inflammation, as judged by footpad swelling, as well as joint destruction, as judged by histological analysis of the joints (145). Analogous results have been obtained using IgG-TNF-receptor fusion protein as an inhibitor of TNFα activity (144, 146, 147). The fusion proteins used and the monoclonal antibody (TN3) are likely to have blocked murine lymphotoxin as well as TNFα. The effects of blocking IL-1 have also been studied in CIA using several approaches. Wooley reported using daily injections of IL-1 receptor antagonist, which resulted in a delay in onset and a reduced incidence of arthritis (148). More recently (149) the combination of neutralizing rabbit anti-mouse IL-1α and IL-1β administered during the pre- and post-arthritic periods was reported to reduce the incidence and severity of CIA. We have also demonstrated amelioration of established CIA using a neutralizing antibody to the type I IL-1 receptor (R Williams, LJ Mason, RN Maini, M Feldmann, unpublished observation).

More recently, we have started to analyze the kinetics of cytokine expression during the early stages of DBA/1 CIA using immunolocalization studies. A consistent finding to emerge from these unpublished studies is that TNFα is expressed in the lining layer of the synovial membrane at the time of arthritis onset; IL-1 is also detected but at a slightly later stage of the disease (1–2 days after the onset of clinical arthritis). Thus, these results of cytokine/anticytokine treatment in vivo are consistent with the analysis of human RA tissues in vitro, and they can be extended to the whole animal with its greater complexity, including cell recruitment.

Other results have been paradoxical, such as those with TGFβ and IFNγ. As noted previously, the injection of TGFβ into footpads of normal or collagen-injected rats induced an arthritis or accelerated its onset (73, 74), while systemic administration of TGFβ to collagen-injected mice ameliorated CIA (75, 76, 143). These studies indicate the multipotential properties of TGFβ (72) and its differential effects when injected systemically or locally into the joint.

IFNγ is a cytokine commonly believed important in the pathogenesis of autoimmune diseases, a theory first proposed in a hypothesis concerning the mechanism of autoimmunity, in which IFNγ was envisaged as necessary for upregulation of antigen presentation (150). Evidence to support this concept appeared rapidly in human studies (151–153). Transgenic mice overexpressing IFNγ in various tissue sites were found by Sarvetnick and her colleagues to develop autoimmunity in the pancreas, retina, and at the neuromuscular junction (154, 155). Localized overexpression of a single cytokine yielding autoimmunity is not reproduced by other proinflammatory cytokines, e.g. TNFα or IL-1 in diabetes models. The importance of IFNγ as a local mediator of inflammation in RA has also been confirmed in CIA. Thus, local injection of IFNγ into the footpads of collagen type II–immunized mice accelerates the onset of arthritis and increases the severity of disease (156). Conversely, systemic administration of IFNγ ameliorates CIA, and treatment with anti-IFNγ may exacerbate or ameliorate the disease depending on the mAb used and the timing of treatment (157, 158). The reasons for this are not clear, but IFNγ has a multitude of effects; some such as downregulation of B cell activity or the antagonism of IL-1–induced bone resorption (159) may be beneficial. It will be interesting to evaluate whether IFNγ or IFNγ receptor knockout mice, backcrossed onto DBA/1 mice, will be able to develop CIA.

The most studied transgenic model of arthritis is in mice expressing a modified human TNFα transgene, with the AU-rich 3′ untranslated region replaced by that of β globin (160). These mice develop an erosive arthritis that can be prevented with anti-human TNFα antibody. The arthritis is the major pathology in this mouse, and it is not yet understood why the joints are so sensitive to TNFα, as there is disregulated expression of TNFα in all tissues. The arthritis does not appear to be lymphocyte dependent, because arthritis develops in TNFα transgenic mice backcrossed to RAG knockouts, which do not possess functional lymphocytes (D. Kioussis, personal communication). The spontaneous appearance of arthritis in multiple strains of human TNFα transgenic mice provides strong supporting evidence for the pathogenic relevance of TNFα in driving the arthritis process in vivo.

We have studied cytokine expression in the joints of these huTNFα transgenic mice by culturing synovial cells obtained from inflamed joints. The technique

chosen was dissociation of synovial tissue cells and subsequent culture in the absence of extrinsic stimulation, mimicking the work with human synovium. Abundant human TNFα in a bioactive form was detected, but paradoxically very little or no murine TNFα was seen, suggesting that autocrine TNFα stimulation was not important. However, upon stimulation with LPS, such synovial cell cultures will produce murine TNF. IL-6 was also abundant, as was IL-10, but only a little IL-1 was detected. As human TNFα reportedly binds the murine p55 TNF-R and not the p75 TNF-R, these findings suggest that signaling through the p55 TNF-R is in itself sufficient to produce autocrine and paracrine cytokine induction and to initiate the disease process (D Butler, A-M Malfait, RN Maini, M Feldmann, FM Brennan, unpublished observation).

In human RA synovial cultures, TNFα is the major stimulus for IL-1 production (28). In the mice transgenic for human TNFα, therapy with a neutralizing antibody to the IL-1 receptor (type I) is protective (161), as is the anti-TNFα antibody as originally described (160). This suggests that much of the activity of TNFα in this in vivo model is mediated by its capacity to generate IL-1 or to synergize with it.

ROLE OF CYTOKINES IN DIFFERENT ASPECTS OF ARTHRITIS

Role of Cytokines in T Cell Function in RA

There are abundant T cells in a RA joint, averaging 20–30% of the mononuclear cells in the synovium. Most are CD4$^+$ CD45RO$^+$. There is little evidence for local proliferation, so that continuous recruitment is important. Recruitment involves upregulation of adhesion molecules, found in synovial endothelium, and expression of chemokines, also found there. The cell surface phenotype of the T cells is consistent with chronic activation, with > 50% expressing DR, VLA-1, VLA-4, VLA-7, but < 10% usually expressing IL-2R. T cells do not survive in the absence of stimulatory signals from the T cell receptor or cytokines. Cytokines present in the joint that may be important in sustaining T cell survival and function include IL-2 (low amounts), IL-7, and IL-15. The latter is relatively abundant in RA joints (162). IL-10 reportedly prevents apoptosis in B lymphocytes and T lymphocytes (163, 164), and as it is abundant in RA joints it may have a role in sustaining the survival of T cells there, although its inhibitory effects are also expressed (see previously).

T Cells in Rheumatoid Joints Are Enriched in Th1-Like Cells

A number of T cell cloning studies have been performed in RA, although none is very detailed. In all of them, the production of IL-4 was not a common event,

so that the majority of the cells were Th1-like, producing IL-2 and IFNγ (87, 165). In the mouse, IL-10 is produced by Th2 cells (166), but in RA joints, T cells produce large quantities of IL-10 (85), consistent with previous studies with human T cells in which IL-10 was found in both Th1 and Th2 subsets (167). The preponderance of Th1 cells in the joint would be in keeping with the presence of the major inducer of Th1 cells IL-12 being detectable in small but bioactive quantities (unpublished data).

Which Cytokines Are Responsible for the Damage to Cartilage and Bone?

In rheumatoid arthritis, the destruction of cartilage and bone occurs by erosion mainly at the junction of cartilage, bone, and synovium, a region known as the *pannus* (14). This destruction progressively invades the bone and spreads over the cartilage, occurring in two forms, one highly cellular, expressing essentially the same mixture of cytokines as in the active synovium and associated with active erosion. Another subset is relatively acellular and expresses chiefly TGFβ; it appears to be a site of repair rather than destruction (168). Normal cartilage cells can produce a wide spectrum of cytokines (168), including IL-1, TNFα, IL-6, and can respond to these also. Hence it is not clear which cytokines are important in cartilage damage, although IL-1 and TNFα are clearly implicated as they induce destructive matrix metalloproteinases, such as MMP-1 (collagenase) and MMP-3 (stromelysin), that are involved in cartilage destruction (104, 169, 170), and they also induce bone resorption in in vitro cultures (105, 171). There is increasing evidence that IL-6 may inhibit bone formation and induce bone resorption through its stimulatory effects in osteoclasts (172). Thus it is interesting that IL-6 gene knockout mice do not develop erosions of bone (173). In view of the fact that IL-6 signals via gp130 (128), a receptor chain shared with other cytokines including LIF, IL-11, and oncostatin M, it is conceivable that they may also be involved in bone destruction in RA. IL-11 is a considerably more potent inhibitor of bone nodule formation than is IL-6 (174), whereas in contrast, there is evidence to suggest that LIF may stimulate bone formation (175). Little is currently known about the effect of oncostatin M on bone metabolism, or indeed whether IL-11 or LIF induce bone resorption.

Are the Pathogenesis of Synovitis and Joint Destruction Equally Dependent on the Same Cytokine Network?

A key question is whether the destruction of connective tissue, the major source of long-term joint problems in RA, is driven by the same cell interactions and cytokines as are implicated in the synovitis. This question has been raised by several workers in the field (176, 177); for example, Bresnihan noted that

conventional therapy while improving synovitis has less effect on bone erosion according to the X-ray progression.

Therapeutic trials of anticytokine and other new therapies have the potential to resolve this issue, by demonstrating that effective therapy resolves the synovitis and protects the joints from destruction. All the anti-TNFα clinical trials so far are too short term to assess for joint protection radiographically, but the reduction in pro MMP-3 production reported suggests that joint protection may occur with long-term reduction of TNFα (178).

Neovascularization in RA

The rheumatoid synovium is a much more cellular mass of tissue than the normal synovium and is characterized by an abundance of blood vessels, thus increasing the delivery of cells and molecules to areas of inflammation (179). The neovascularization that takes place in RA is central for the maintenance of the disease process, since inhibition of angiogenesis using either the microtubule stabilizer Taxol or AGM-1470, a synthetic angiogenesis inhibitor, blocks arthritis in a rat model of CIA (180, 181).

The process of neovascularization in RA involves angiogenic cytokines such as VEGF, an endothelial cell-specific mitogen that promotes the growth of new blood vessels (182) and renders the vasculature hyperpermeable in vivo (183). VEGF has been detected in RA joint tissue and synovial fluids by two independent groups (those of Fava and of Koch) and was associated with strong expression of VEGF mRNA by synovial tissue macrophages and of VEGF receptors by microvascular endothelial cells (184, 185). Hypoxia is a strong inducer of VEGF release and VEGF receptor expression (186, 187); the RA joint reportedly is hypoxic due to pressure on vessels during movement (188), although other inflammatory stimuli within the RA joint may also contribute to VEGF production from macrophages. In this respect, it is interesting to note that TGFβ has recently been found to induce VEGF release from RA synovial cells (R Fava, unpublished observation). VEGF also is chemotactic for endothelial cells, which may perpetuate angiogenesis.

Finally, it has been reported that a large number of cytokines elevated in RA, including TNFα, TGFβ, FGF, as well as chemokines such as IL-8 (189), and possibly other C-X-C chemokines such as GRO α and ENA-78 (S Kunkel, unpublished observation) may also exhibit angiogenic activities.

The Cytokine Network in Other Forms of Arthritis

Compared to rheumatoid arthritis, little is known of the cytokine network in other forms of arthritis. Due to the availability of operative samples at joint replacement, osteoarthritic (OA) synovium has been used as a control for many of the studies in RA. It is not, however, a good comparison, because the diseases

differ in their underlying etiology: RA has an autoimmune component, while OA does not. By the stage of joint replacement, it is evident that an extensive phase of tissue destruction has occurred in OA, with much of this attributable to an inflammatory component. This is perhaps why the cytokine pattern appears to be relatively similar to that in RA. There is no clear-cut distinction, as the cytokines TNFα, IL-1, and IL-6 are all expressed, albeit at lower average levels (28, 29). Chemokine expression has also been noted, including that of IL-8 (53) and MCP-1 (57). Anti-inflammatory cytokines such as IL-10 (67) and TGFβ (69) are abundantly expressed in OA, as in RA. Perhaps one of the few differences is in the bioactivity of TNFα in synovial cultures. While all RA synovial cultures produce bioactive TNFα, a significant percentage of OA synovial cultures do not. This is due to excess free sTNF-R (125). For most of its long history, OA has had a tendency to induce excessive bone formation (e.g. osteophytes), which must involve a cytokine pattern different to that in RA. What prompts the change to a bone destructive form of OA would be interesting to understand, due to its therapeutic implications. Other forms of arthritis such as psoriatic arthritis and infection-related reactive arthritis have not been well documented at the cytokine level.

The arthritis in reactive arthritis, in contrast to RA, is not destructive, and as such synovial tissue is much harder to obtain. One interesting comparison has been performed at the mRNA level by Simon et al (86). Reactive arthritis specimens often produced IL-4 mRNA (6/8), but only 2 out of 10 of those from the rheumatoid did, even if analyzed by RT-PCR. This admittedly limited study suggests that the Th1/Th2 balance may be critical for a self-limiting arthritis, and the excess of Th1 cells in rheumatoid arthritis may be important in its persistence (NA Mitchison, unpublished).

LESSONS FROM CLINICAL RESEARCH AND TRIALS

Cytokine Antagonists as Therapeutic Agents

In the previous sections we have analyzed the experimental evidence for the evolving concept that the broad spectrum of cytokines locally produced in joints provides a molecular basis for the observed pathology. Now we examine the extent to which it has proven possible to validate the role of each cytokine in vivo by using specific antagonists of high affinity and specificity such as monoclonal antibodies, soluble cytokine inhibitors, and in the case of IL-1, IL-1ra, in the therapeutic setting. The accumulating evidence suggests that TNFα is not only an inflammatory mediator in its own right but also is the key regulator of the production of other cytokines implicated in rheumatoid inflammation such as IL-1 (28), GM-CSF(34), IL-6 and IL-8 (109). We have therefore espoused

TNFα as a prime target for therapeutic trials. However, antagonists of IL-1 and IL-6 have also been tested in the clinic by others; in the case of IL-1 antagonists, the results of randomized clinical trials have not yet been published in full, and anti-IL-6 therapy has thus far been tested only in an open-label trial.

Anti-TNF Clinical Trials

The availability of a chimeric (mouse × human) monoclonal anti-TNFα antibody cA2 (from Centocor, Malvern, Pennsylvania), with an affinity of 1.8 $\times 10^{-9}$ kDa and with neutralizing activity on a variety of biological activities of TNFα in vitro (190), provided an opportunity for testing hypotheses for a new treatment of RA (191, 192). Chimerization of the antibody (75% human immunoglobulin) should reduce immunogenicity and increase the half-life of murine antibodies (193). This monoclonal antibody (cA2) had been shown to be nontoxic in preclinical safety tests on monkeys and human volunteers in the dose range that was predicted to be therapeutic, based on the dosage needed to ameliorate collagen-induced arthritis in DBA/l mice (145).

Accordingly in 1992/1993, in an open-label trial, we administered cA2 by two to four intravenous infusions over two weeks, in a total dose of 20 mg/kg body weight, to 20 patients who had chronic erosive RA unresponsive to multiple disease modifying drugs (DMARDs) such as gold, methotrexate, and salazopyrine (194). To avoid the confounding carry-over effects of previously administered DMARDs and of simultaneous ongoing anti-inflammatory therapy, patients ceased DMARDs for a minimum of four weeks and were stabilized on a fixed dose of nonsteroidal anti-inflammatory drugs (NSAIDs) and/or corticosteroids in this period. The treatment with anti-TNFα monoclonal antibody led to rapid improvement in every patient in all indices of disease activity used for monitoring patients. Such modalities included clinical evaluations such as the number of swollen and tender joints, the degree of pain experienced, the duration of stiffness of joints on waking in the morning, locomotor function, the physician's assessment of overall benefit, and the patient's assessment of improvement. Laboratory measurements of inflammatory activity such as the erythrocyte sedimentation rate (ESR) and C-reactive proteins (CRP) also showed marked reduction. The improvement from baseline reached its maximum within the first four weeks, with mean values of change exceeding 60–70%, and lasted 8–22 weeks (median 12 weeks). Since the trial was primarily to test the safety of the therapy, particular attention was paid to vital signs and side effects during and following therapy. The infusions were tolerated without any change in cardiorespiratory function or body temperature, and patients seemed to be free of adverse events during the trial period.

The magnitude of clinical response and reduction in CRP was convincingly reproduced in a randomized, double-blind, multicenter, placebo-controlled trial

on 73 patients in 1993/1994 in four European centers (195). In this trial, entry requirements were active erosive rheumatoid disease and a history of failure to respond to at least one DMARD. Having withdrawn from DMARDs, patients were stabilized on a fixed dose of anti-inflammatory drugs for four weeks. Thereafter, a single intravenous infusion of cA2 at two doses (1 mg/kg or 10 mg/kg) or placebo infusion (0.1% human serum albumin in normal saline) was administered over 2 hr to 24 patients in each group (25 in the 1 mg/kg group because of premature dropout of one patient in the other). An impressive change was seen in all measurements and is illustrated in Figure 3 for the number of swollen joints and CRP. The primary endpoint of this trial was the achievement of improvement in a composite index of disease activity defined by Paulus et al (196), which discriminates between DMARDs and placebo effects in randomized controlled trials. According to this index, a positive result was defined as significant improvement in four out of six variable measurements defined as: 1. at least 20% improvement in continuous variables (tender and swollen joint scores, duration of morning stiffness, and ESR); 2. at least two grades of improvement in patient's and observer's assessment of disease activity.

When the Paulus criteria were applied in the trial, a striking benefit was observed in the majority of patients treated with anti-TNFα when compared with those treated with a placebo (Figure 4). According to the predetermined primary endpoint of the study at four weeks, 79% of patients significantly improved, compared with 44% at the lower dose. In contrast, 8% of patients receiving placebo achieved the same level of response at 4 weeks. In order to define

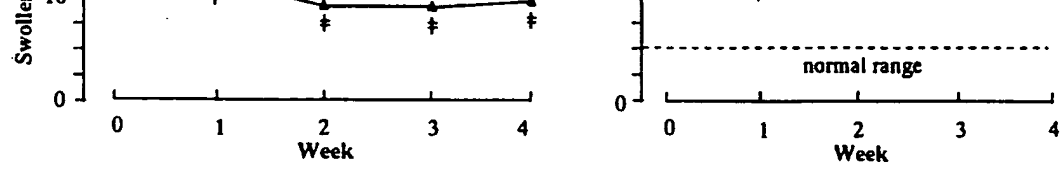

Figure 3 Swollen joint count assessment and C reactive protein measurement in patients receiving placebo (● or cA2 anti-TNF mab at 1 mg/kg (♦), and 10 mg/kg (▲). Values are means of 24 patients at each point (25 for 1 mg/kg group). Significance versus placebo: *p < 0.05, †p < 0.01, ††p < 0.001. Reprinted by kind permission of *The Lancet* (Elliott et al, 1994, 344: 1105–10).

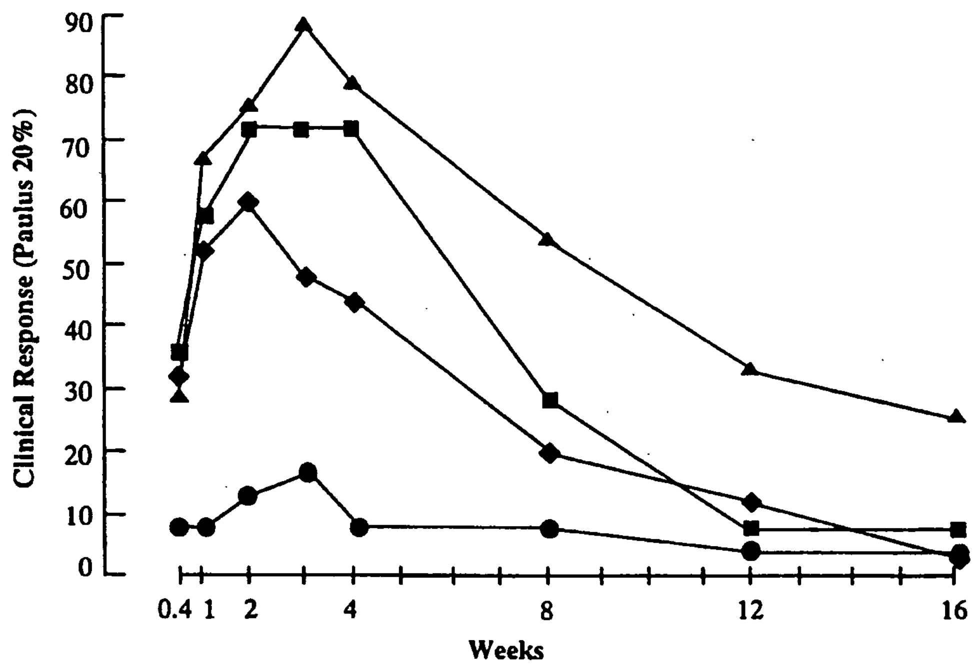

Figure 4 Duration of clinical response assessed by the Paulus disease activity index following placebo, 0.1% HSA, $n = 24$ (●); and cA2, anti-TNF mab at 1 mg/kg, $n = 25$ (♦); 3 mg/kg, $n = 14$ (■); and 10 mg/kg, $n = 24$ (▲). Based on data in (Elliott et al, 1994, 344: 1125–27).

the proportion of patients who showed an even more marked improvement, the Paulus Criteria were arbitrarily reset by increasing the threshold of response to 50% improvement in continuous variables, and the data were reexamined. Using these rigorous criteria, 58% of patients benefited at the high dose of anti-TNFα monoclonal antibody and 28% at the low dose (195). This profile of data at four weeks seemed outstanding when compared with the effect of other drugs tested in randomized placebo-controlled trials.

Analysis of the dose-response relationships of parameters of disease activity in patients revealed that in the initial two weeks, the proportion of responders at the low dose (1 mg/kg) matched that at high dose (10 mg/kg). The magnitude of response in the individual clinical parameters of disease activity (such as number of swollen and tender joints, ESR, and CRP), generally showed a 60–70% change from baseline. The difference in response between the low and high doses lay in the duration of the response.

The duration of response was further examined by following all treated patients to relapse of disease. Upon relapse, a further infusion of anti-TNF was given. According to protocol, 14 patients who had initially received placebo then subsequently received an intermediate 3 mg/kg dose of anti-TNFα. This permitted an evaluation of the duration of response at three doses of anti-TNF.

The median duration of response was clearly dose-dependent (Figure 4), lasting 3 to 8 weeks in this dose range and persisting for over 16 weeks in 25% of patients receiving the high dose of anti-TNF antibody (197).

The pharmacokinetics of the antibody have given insight into the duration of response. Preliminary analysis shows that the Cmax following single infusions of the 1 mg/kg and 10 mg/kg of cA2 were 28 mg/L and 328 mg/L, respectively. The kinetics appear to fit a two-compartment model. Using the mean serum concentrations over time following a 10 mg/kg dose, calculations show that the half-life of the antibody is around 18 h (at one week). More significantly, in interpreting clinical responses, detectable levels persist for 10–12 weeks at the high doses. At lower doses the antibody persists for a correspondingly shorter duration (197). It is of interest that at 1–5 mg/ml of cA2, TNFα effects in rheumatoid joint cell cultures are inhibited (109). Levels above 1–5 mg/ml are maintained depending on the dose administered. It explains why the initial magnitude of the response and proportion of patients is the same for the three doses used, but diverges with the passage of time and a fall in circulatory level below the therapeutic threshold.

Since the efficacy of a single treatment cycle has a finite duration of several weeks and because the disease relapses in all cases, the question arises whether the anti-TNFα monoclonal antibody therapy is efficacious in controlling chronic disease if given in repeated infusions. Only limited data are available currently, although an ongoing study on 105 patients receiving repeated cycles will attempt to address this point. However, what is known from the experience gained in retreating seven patients with up to four cycles of anti-TNFα monoclonal antibody following relapse is that it was possible to use the antibody repeatedly and to demonstrate an equivalent magnitude of control of disease with subsequent infusions, although there was a trend toward a shortening of effect in some patients (198). The results encourage the view that TNFα blockade is likely to be beneficial as long-term therapy. There is as yet no evidence for the emergence of a TNFα-independent pathway, which could have been the case in view of the potential redundancy of the cytokine network with numerous proinflammatory mediators.

Since TNFα is known to be of importance in host resistance to infections, we have taken an interest in documenting infective events in treated patients. However, RA patients are liable to infections to a greater extent than the healthy control populations (199) and may be influenced by a reporting bias under continuing close scrutiny. The only accurate way of assessing an increased incidence is to compare the type and rate of infections against a population of RA patients stabilized on equivalent background therapy, but not receiving antiTNFα monoclonal antibody. This has been possible in the randomized

controlled study in the first four weeks, and results showed an incidence of six episodes in 49 patients, or 12% (three upper respiratory, two skin infections, and one episode of pneumonia in anti-TNFα treated patients) versus one upper respiratory tract infection in 24 patients (4%) receiving placebo therapy. The significance of these predominantly minor infections will require further analysis following a more extensive experience, but the result does not suggest that it is a limitation of therapy. The observation of a trend toward normalization of indices of previously depressed cellular immune functions common in RA patients (200–202) following anti-TNFα therapy, such as lymphocyte proliferation in vitro (203) and delayed hypersensitivity reaction to recall antigens in vivo (204), is reassuring in this context.

An unexpected side effect in these clinical trials was the development of IgM class anti-ds-DNA antibodies some weeks following cA2 treatment in 7 out of 91 patients (6%) without evidence of clinical SLE. Three of these seven received another infusion, and in only one did the titer of antibody rise; in one it remained unaltered; and a third showed reversal to normality. In all patients the antibody levels spontaneously resolved over several months. The pathogenesis of anti-ds-DNA antibodies in a minority of RA patients is unexplained at this point, but it is noteworthy that other anti-rheumatic drugs can induce ds-DNA autoantibodies (205). The development of lupus serology following TNF depletion is consistent with observations made by Jacob and McDevitt in NZB/W mouse model of SLE, in which TNF deficiency was associated with a genetic polymorphism of the TNF locus in this mouse (206). More recently, in anti-IL-10–treated NZB/W F_1 mice (207), remission was attributed to induction of TNF, since anti-TNF therapy caused rapid recurrence of severe disease. It would appear that in a subpopulation of RA patients, anti-TNFα may similarly predispose to anti-ds-DNA antibodies, and there is a risk that a proportion of these patients might develop a lupus-like syndrome with long-term therapy. These studies suggest that the cytokine network is fundamentally different in RA and SLE (208)

Trials of TNFα blockade with other biological agents have been reported by other groups. In a double-blind dose-ranging study, a humanized mouse monoclonal antibody (CDP 571, Celltech, Slough, UK) was administered to 24 patients in a single dose at 0.1, 1, and 10 mg/kg (209). In this antibody the hypervariable regions were grafted into a human IgG_4 immunoglobulin. The results when compared with 12 placebo-treated patients showed that it was well tolerated, and significant improvement appeared in a composite disease-activity score and in some of the individual disease-activity criteria. Unlike with cA2, however, no significant difference was noted in the swollen joint score. Reductions in CRP were also observed, thus confirming the results with

cA2, but the comparative dose-response relationships suggest that the potency of CDP571 was less than that of cA2. Whether this reflects differences in epitope specificity, affinity, or isotype (IgG_4 versus IgG_1) is unknown, but further investigation could illuminate the mode of action of the two anti-TNFα antibodies. CDP571 also induced anti-ds DNA antibody production (D. Isenberg, personal communication, European Rheumatology Meeting, 1995).

Engineered soluble TNF receptors linked to Fc portion of IgG, which are divalent and act as potent inhibitors of TNF (210), are being developed for therapy of disease by the pharmaceutical companies. Such soluble TNF receptor fusion proteins should be less immunogenic than monoclonal antibodies partly derived from mice, and they may be therapeutically active, since they bind TNFα with high affinity. The bioengineered immunoglobulin-like molecule might also be expected to increase its circulating half-life. Two such products are undergoing therapeutic trials in RA patients. A p75 soluble-TNF-R, linked to Fc of IgG_1 (Immunex), or placebo, has been administered in a Phase I study to 16 patients with refractory RA at the University of Alabama at Birmingham (211, 212). Three patients in each group received an intravenous loading lose of 4, 8, 16, and 32 mg/m^2, followed by subcutaneous injections twice weekly for four weeks, of 2, 4, 8, and 16 mg/m^2 in the four groups, respectively. Additionally, four patients received placebo injections. No serious adverse events were recorded. Clinical responses did not show a dose-response improvement, but at day 31 following commencement, there was a 40–55% mean improvement in painful, swollen, and tender joints in anti-TNFα-treated groups compared with 22 to 25% improvement in the placebo group; however, these differences were not statistically significant. CRP was reduced by 25% compared with 13% in the placebo group, and this also was not statistically significant. Roche has used a p55 soluble TNF-receptor IgG_1 fusion protein in ongoing trials but has not disclosed data, though abstracts and meeting presentations claim efficacy.

We may conclude from these trials with several different agents that TNFα antagonists are likely to be beneficial in RA, but the precise indications for their use and their limitations as a generic therapy or the relative efficacy of individual biological variants must await further development. The current data are short term, and assessment of long-term safety will need further clinical trials.

Mechanism of Action of Anti-TNFα Antibody Therapy

Rheumatoid inflammation results from the conjoint effects of multiple mediators locally produced by many cell types. We have presented evidence based on clinical trials of the marked dose-dependent anti-inflammatory effects of anti-TNFα therapy. However, it remains to be established which of the many biological effects of TNFα (213) is of central importance in amelioration of symptoms and reduction of acute phase proteins. Our current hypothesis suggests two

main mechanisms of action: first, local deactivation of the proinflammatory cytokine cascade following neutralization of excess amounts of TNFα at the inflammatory site; and second, an interruption of cellular recruitment and traffic into rheumatoid joints as a result of a reduction of TNFα-dependent expression of vascular adhesion molecules, and of chemokines.

The remarkable reduction in levels of acute phase proteins (e.g. CRP) following within 72 h of infusion of anti-TNFα antibody cA2 (194) indirectly points to an effect on the cytokine network. The production of CRP by the hepatocyte is known to be regulated by cytokines, chiefly by IL-6, but also by other cytokines such as LIF and IL-11, signaling via the gp130 receptor. We have measured serum IL-6 levels before and after infusion of anti-TNFα antibody, and they have shown a rapid decline that parallels a fall in the CRP concentration in the blood (194, 197, 214). Predictably, the subsequent rise in CRP is also closely associated with an increase in IL-6 levels, occurring earlier at the lower dose (1 mg/kg) when compared with the higher dose (10 mg/kg) of anti-TNF. A reasonable explanation of this sequence of events is that neutralization of TNFα in the synovial compartment interferes with the cytokine network (see earlier), thereby reducing IL-6 production as occurs in rheumatoid joint cell cultures (109). Cytokine deactivation is mirrored by a fall of IL-6 levels in blood and is followed by a reduced end-organ response, as judged by a fall in CRP synthesis by the hepatocyte.

Cytokine blockade and diminution of other downstream inflammatory pathways is probably of major importance in the rapidity of reduction in pain, stiffness, swelling, and tenderness of joints. The best objective evidence of an anti-inflammatory effect currently available is based on histopathological examination of serial biopsies of synovial tissue obtained from multiple sites of knee joints before infusion with anti-TNFα antibody and 14 days later. Examination of coded multiple sections of synovium by two blinded observers overcame observer bias and variability of the cellular response in different regions of the joint, and results clearly showed a reduction in the cellularity of synovium (197, 215). Reduction is seen in the number of cells/high power field in the synovial lining layer, infiltrating mononuclear cells (predominantly macrophage-like cells), and perivascular lymphoid aggregates.

The reduced cellularity of synovium also supports our second hypothesis, suggesting a reduction in trafficking of blood-borne cells into diseased joints. A simultaneous significant increase in the number of circulating lymphocytes accompanying a fall in synovial infiltrate is in keeping with reduced influx into joints. That this is dependent on TNFα-modulated expression of adhesion molecules is supported by the decreased expression of ICAM-1, VCAM-1, and E-selectin in the biopsies (197, 215, 216). ICAM-1 and VCAM-1 are

detected at vascular endothelial sites in the synovium as well as in many of the lining layer cells. E-selectin was detected on up to 28% of vascular endothelial cells of capillaries and venules. Following anti-TNFα therapy, we observed a significant reduction in E-selectin expression in endothelium and also an overall reduction in VCAM-1 and ICAM-1 expression in the lining layer.

Recently, the detection of soluble forms of the adhesion molecules ICAM-1, VCAM-1, and E-selectin in blood has become possible by ELISA. These molecules are derived from proteolytic cleavage of the surface form (217), and their release correlates with increased surface expression following activation by TNFα and IL-1 on cultured endothelium (218, 219). Elevated serum levels of ICAM-1, VCAM-1, and E-selectin have been observed in RA, and soluble VCAM-1 and ICAM-1 appear to correlate with disease activity (220, 221).

In our European multicenter, randomized placebo-controlled study with anti-TNF antibody, we showed a dose-related reduction in soluble E-selectin and soluble ICAM-1 levels (221), but interestingly not of VCAM-1 levels. The reduction paralleled parameters of clinical response, ultimately returning to pretreatment levels closely in parallel with recurrence of symptoms of inflammation. It was noteworthy that in cA2-treated patients (1 mg/kg and 10 mg/kg pooled), there was very good correlation between clinical response and reduction in serum E-selectin (221). Recruitment of cells into the synovial tissue involves activation of cell surface integrins such as LFA-1 and ICAM-1, and chemokines are powerful signals for upregulating the affinity of these integrin molecules. Interestingly, anti-TNFα in vitro diminished IL-8 production (109), and preliminary evidence indicates that this also occurs in vivo. It is thus very likely that the diminished recruitment of cells into joints partly explains the prolonged duration of clinical benefit.

In conclusion, the reproducible clinical benefit of TNFα blockade in RA using antibodies has clearly underlined the importance of understanding the role of cytokines in arthritis. It may not be farfetched to consider that treating earlier in the diseases process, or with longer term TNFα blockade, it may be possible to redress the homeostatic balance and obtain longer term benefit (remissions) and possibly cures in some patients.

IL-1 and IL-6 Antagonists

Recombinant human interleukin-1 receptor antagonist (IL-1ra), manufactured by Synergen, is a candidate drug for use in RA since it successfully blocks the effects of IL-1 (130). The requirement for 90% receptor occupancy to achieve IL-1 blockade means that the agent has to be used repeatedly at relatively high dose levels as the molar ratio of IL-1ra to IL-1 needs to be >10. In a randomized double-blind controlled trial, 175 patients with RA were divided into nine groups that received 7, 20, or 200 mg, once daily, three times a week,

or once weekly for three weeks. Subsequently all patients received a dose once a week for four weeks. Of these patients 14% withdrew due to lack of efficacy or adverse events. Data in abstract form (222) shows that at three weeks patients receiving daily injections showed significant improvement compared with patients receiving weekly injections, but no dose-response relationship was apparent in patients receiving the three-dose levels of daily injections. Significant but relatively modest reduction in CRP (< 40%) was observed. The effects apparently lasted three to seven weeks in some patients.

Recombinant type I soluble IL-1 receptor (sIL-R, from Immunex), which inhibits the binding of IL-1 to its cell-associated receptor, has been used in a Phase I randomized single center study utilizing intra-articular injections in the knee. Four doses were evaluated in four RA patients in each group (total 16) and compared with a placebo injection in four patients. Following 25, 100, 250, and 500 mg injection into a knee, a dose-related reduction in knee circumference was observed at 48 h and 7 days, reaching significance only at the dose of 250 mg (223).

In another randomized, double-blind study reported in abstract form, sIL-1R was administered by daily subcutaneous injection for 28 days. Twenty-three patients with RA were treated with 125, 250, 500, and 1000 mg/m^2 daily and compared with patients receiving a placebo. No patient met predetermined criteria for significant improvement (224). In this study the choice of a type I soluble IL-1 receptor (rather than type II) may not have been optimal, since it binds IL-1α with greater affinity than does the predominant IL-1β form secreted in RA joints. It also preferentially binds endogenously produced IL-1ra in joints, which thus may negate its potential benefit, and possibly increases the bioavailability of IL-1β by displacement from sIL-R (127). The limited clinical trial data available on IL-1 blockade, by the agents described above, do not permit any definitive conclusion concerning the relative merits of blockade of IL-1 and TNFα. Other IL-1 blocking agents or different clinical protocols may yield greater benefit, but at the moment it is not proven that IL-1 blockade is effective in RA; this is in contrast to TNFα blockade by monoclonal antibodies, in particular cA2 (194, 195, 209).

A murine anti-IL-6 monoclonal antibody has been administered in an open-label study to eight patients with refractory RA (225, 226). Improvement in pain and number of tender joints was observed, lasting two months. An impressive fall in CRP and ESR occurred in this period. The authors report an increase over baseline in circulating levels of immunoreactive IL-6 following treatment. The significance of this trial and IL-6 data is unclear, but it merits further investigation.

CONCLUDING REMARKS

In this chapter we have attempted to encapsulate the impact of cytokine research in providing fresh insight into molecular mechanisms of disease. These developments have been dependent in equal part on the availability of a novel array of biological agents and their application, as specific antagonists or agonists, in experimental model systems and clinical trials. Interim conclusions can be drawn and give rise to cautious optimism on the potential of these experimental approaches in unravelling complex biological responses and assessing the therapeutic value of neutralizing or augmenting specific cytokines in the clinical situation.

Among the encouraging results is the demonstration that monoclonal antibodies and other biological products that act as specific cytokine antagonists can be administered to patients safely, at least in the short term. Despite the pleiotropy intrinsic to the action of many cytokines, it appears that their blockade can lead to impressive clinical benefits—for example, following anti-TNF therapy. Moreover, repeated administration leads to reproducible effects, arguing against redundancy in the biological systems involved in disease. However, complete and sustained remission of disease has not been achieved, and it is unclear whether current modes of long-term delivery of biologicals will be tolerated without evoking allergic responses in a proportion of patients or inducing immune responses, which by neutralizing their biological activity will lead to loss of efficacy of the therapeutic principle. It is also not yet known whether long-term neutralization or augmentation of the activity of a cytokine may be associated with unanticipated side effects. More experience is clearly necessary, although the speed of progress is such that availability of biologicals as licensed drugs in the treatment of arthritis in two to three years is a distinct possibility. In the meantime, improved systems of delivery of biologicals, for example, by gene therapy, may provide exciting prospects for development.

Among the challenging questions to be addressed in the near future is the possibility that better results may be obtained in controlling RA by combination therapy. Thus, more than one cytokine might be targeted simultaneously, or proinflammatory cytokine blockade may be combined with anti-T cell therapy. An equally compelling case can be made for testing biologicals in combination with existing disease-modifying drugs of proven efficacy, such as methotrexate and salazopyrin. Whichever approach is used, the high frequency of placebo responders in RA makes the testing of drugs in randomized controlled trials mandatory. The equally pressing need to use validated criteria of benefit in clinical trials is being addressed by co-operation at international level. These efforts should simplify the task ahead and allow comparisons to be made.

The definition of therapeutic targets by the use of biologicals has stimulated the reevaluation of mechanisms of action of established drugs, and it has also stimulated the search for chemical drugs that carry the specificity of biologicals. The race between the approaches has begun, and the potential strengths and weaknesses of each one are all too apparent. So are the enormous opportunities to improve significantly on the limited benefit of drugs in primary and secondary prevention of disabling arthritic disorders.

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Opinion

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CD69 is an immunoregulatory molecule induced following activation

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CD69 is an early leukocyte activation molecule expressed at sites of chronic inflammation. The precise role of CD69 in immunity has not been elucidated owing to the absence of a known ligand and adequate *in vivo* models to study its physiological function. Although previous *in vitro* studies suggest that CD69 is an activatory molecule in different leukocyte subsets, recent studies in CD69-deficient mice have revealed a non-redundant role for this receptor in downregulation of the immune response through the production of the pleiotropic cytokine transforming growth factor-β (TGF-β). The possible cellular and molecular mechanisms of action of this molecule are discussed herein.

Introduction

Self-limitation of the immune response is crucial to its control and molecules induced during lymphocyte activation might act as negative regulators. In this Opinion, we discuss recent results that identify CD69 as a potential negative regulator. CD69 is an early membrane receptor transiently expressed on lymphocyte activation, not detected in resting lymphocytes, and selectively expressed in chronic inflammatory infiltrates and at the sites of active immune responses *in vivo*. Although early *in vitro* data suggested that CD69 exerts a proinflammatory function, recent *in vivo* results indicate that this receptor might act as a regulatory molecule, modulating the inflammatory response. In addition, CD69 might act specifically on an as yet uncharacterized T-cell regulatory subset. These recent insights provide a novel view of the function of this receptor, even though a full picture of the spatial and temporal regulation of the immune response by CD69 will require detailed characterization of its ligand(s).

Early data: CD69 exerts a co-stimulatory effect *in vitro*

The *CD69* gene is located within the natural killer (NK) gene complex on mouse chromosome 6 and human chromosome 12 [1,2] and codes for a type II C-type lectin ascribed to the family of NK receptors. CD69 is expressed following activation in all bone marrow-derived cells except erythrocytes (reviewed in Ref. [3]). Most NK lectin receptors directly mediate their activatory or inhibitory effects through their cytoplasmic domains [4]. However, the cytoplasmic domain of CD69 is short and lacks any identifiable function-associated motifs. Thus, no signalling proteins have been described that directly associate with its cytoplasmic domain, although recent results show that CD69 activates Syk in a Src-dependent manner in NK cells [5]. These tyrosine kinases control downstream activation of phospholipase Cγ2 (PLCγ2) and Vav1 that, in turn, activate the Rac–ERK (extracellular signal-regulated protein kinase) pathway [6], which is implicated in NK-cell activation. In addition, some studies have reported the involvement of a CD69-coupled heterotrimeric G protein in its intracellular signalling pathway [7–9].

The rapid and transient induction of CD69 expression on T cells suggests that it might enhance activation and/or differentiation, as occurs with CD40L (CD154) or CD25. In the absence of a known ligand, *in vitro* studies to dissect the possible function of CD69 were based on the use of specific monoclonal antibodies (mAbs) (reviewed in Ref. [3]). In the presence of phorbol esters, anti-CD69 mAbs stimulate the production of interleukin-2 (IL-2), which increases T-cell proliferation [10,11], and tumour necrosis factor-α (TNF-α) synthesis [9,12], whereas they induce nitric oxide (NO) secretion by monocytes [13] and activation of arachidonic acid metabolism and degranulation in platelets [14], suggesting that CD69 could act as a proinflammatory receptor. In addition, cross-linking of CD69 with secondary antibodies mediates early signalling events, such as extracellular Ca^{2+} influx [9–11], relieving the blockade in capacitative calcium entry in antigen-primed T cells [8]. Moreover, antibodies against CD69 significantly inhibit the ability of T cells to activate macrophages by cell contact [15], suggesting that a putative CD69 counter-receptor expressed by macrophages is involved in the production of proinflammatory cytokines. Therefore, CD69 can apparently mediate immune cell activation and exert proinflammatory effects *in vitro* either directly or indirectly. However, CD69 engagement also triggers apoptosis in different cell types, such as monocytes or eosinophils [16,17], and might mediate inhibitory signals on IL-1 receptor (IL-1R)- or CD3-mediated T-cell proliferation [18]. All these data indicate that CD69 behaves *in vitro* more as a co-stimulatory receptor than as a net inhibitory or activatory molecule, although the fate of this co-stimulation could vary depending on the cellular context.

Recent insights: immunoregulatory role of CD69

The *in vivo* models initially chosen for the study of CD69 function were based on its pattern of expression. Studies

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in CD69-transgenic mice focused on thymic selection [19,20], a process in which CD69 expression is transiently induced (Box 1). Despite the *in vitro* evidence suggesting a possible proinflammatory role for CD69, constitutive expression of CD69 by T cells in transgenic mice is not associated with inflammatory conditions [19,20]. Furthermore, analysis of antigen-specific responses in mice has not revealed reduced T-cell activation in the absence of CD69 [21], suggesting that this receptor does not exert a net positive co-stimulatory effect in T cells *in vivo*, although a redundant role as a positive co-stimulus for T cells cannot be ruled out.

Given the somewhat contradictory *in vitro* and *in vivo* results, it became appropriate to study the role of CD69 in an *in vivo* model of chronic inflammation. This analysis was based on two lines of evidence. First, CD69 is persistently expressed at inflammatory foci [22]. Second, the *CD69* gene is located at the Cia3 trait loci on rat chromosome 4 and mouse chromosome 6 [1], syntenic to human 12p12–p13 [2], a region that contains susceptibility loci for several autoimmune diseases, including collagen induced arthritis (CIA) [23,24]. Remarkably, the study of CIA in CD69-deficient mice unveiled a new regulatory role for CD69 (Figure 1). CD69-deficient mice develop an exacerbated form of CIA with higher T- and B-cell responses against collagen [25]. This hyper-responsiveness correlates with reduced levels of TGF-β in inflamed joints (Figure 1). TGF-β acts as an anti-inflammatory cytokine in CIA [26], and treatment with blocking anti-TGF-β antibodies exacerbates arthritis severity, increasing proinflammatory cytokines and chemokines, in wildtype but not in CD69-deficient mice [25]. The reduced levels of TGF-β and the absence of CD69 could be causally associated. In this regard, CD69 cross-linking *in vitro* promotes TGF-β synthesis [25,27]. In addition, TGF-β synthesis is dependent on ERK activation [28] and CD69 cross-linking mediates ERK activation [6]. Hence, the regulatory effects of CD69 *in vivo* appear to be mediated through the synthesis of a pleiotropic cytokine, which might be finely tuned by the controlled expression of CD69 ligand(s).

CD69 cross-linking induces TGF-β production in $CD4^+$ and $CD8^+$ T cells as well as in NK cells and macrophages [25,27], suggesting that this receptor exerts a wide immunoregulatory action, and that other cells, expressing the corresponding counter-receptor, might also participate in this phenomenon. Thus, CD69 could influence not only adaptive but also innate immunity. Accordingly, in an NK-sensitive tumour model in mice, CD69 deficiency leads to reduced TGF-β synthesis by immune cells that results in a high production of chemokines, with decreased lymphocyte apoptosis, accumulation of NK cells and enhanced tumour lysis [27]. Supporting these data, blockade of TGF-β signalling in T cells enhances anti-tumour immunity by facilitating the expansion of tumour-specific $CD8^+$ T cells [29].

Both the NK-sensitive tumour model and the CIA model demonstrate that CD69 deficiency leads to diminished TGF-β levels that support an enhanced immune response, resulting in a more efficient depletion of tumours or increased inflammation in the CIA model [25,27]. The use of an antibody that downregulates CD69 expression *in vivo* reproduced in wildtype mice the phenotype found in CD69-deficient mice [27], further supporting the proposed immunoregulatory role of CD69.

However, as mentioned earlier, CD69 cross-linking *in vitro* also mediates production of proinflammatory mediators [9,12–14], thus suggesting that CD69 could have a dual role, mediating the synthesis of different cytokines, depending on the particular cellular context. It has been reported that CD69-deficient mice are resistant to the induction of granulocyte-mediated acute arthritis, which is initiated by the administration of exogenous anti-collagen II antibodies and endotoxin [30], an inflammatory condition in which the regulatory mechanisms exerted by lymphocytes are not involved. It is feasible that although TGF-β has a predominant inhibitory effect on T and B cells [31,32], this cytokine might act as a chemotactic and activating agent on granulocytes [33], which are the main mediators of this acute model of arthritis. Therefore, reduced synthesis of TGF-β might still account for the attenuated inflammatory response seen in this model. Alternatively, CD69 might affect the synthesis of proinflammatory molecules in a non-redundant way in this particular model, whereas in the CIA model any effect of CD69 on the synthesis of proinflammatory molecules [15] could be compensated by other receptors. The possible dual role of CD69 makes it difficult to predict the final outcome of engagement by its ligand(s),

Box 1. CD69 and thymocyte physiology *in vivo*

CD69 is transiently expressed in thymocytes that are undergoing positive selection or that have just completed this process [47–49]. Positive selection is a multi-stage process involving a first step in which CD69 is induced in double positive thymocytes, a process dependent on MHC molecules expressed by thymic epithelial cells, and a second step to maturation to single positive thymocytes that is MHC-independent [50]. These data indicate that CD69 is a marker of a thymocyte subset that differentiates and proliferates in an MHC-independent fashion and suggest that this molecule mediates this process. However, CD69 deficiency does not affect thymic development and positive or negative selection of thymocytes [21]. By contrast, the constitutive expression of CD69 during T-cell development induces an increase in both CD8 and CD4 single positive thymocytes in thymus medulla [19,20]. These data suggest that the constitutive expression of CD69 does not interfere with thymocyte development but inhibits the export of mature single positive thymocytes to the periphery [19]. By crossing CD69 transgenic mice with different TCR transgenic mice, Nakayama *et al.* showed enhanced negative selection that caused a reduction in the number of T cells in peripheral lymphoid organs [20]. Interestingly, CD69Δcyt transgenic mice, constitutively expressing CD69 without the cytoplasmic domain, show a phenotype similar to CD69 transgenic mice [20], suggesting that the putative CD69 ligand expressed in the thymus is responsible for this phenotype when CD69 is overexpressed. Because CD69-deficient mice show normal thymocyte selection [21], either the putative CD69 thymic ligand acts just as a mechanism of retention of $CD69^+$ thymocytes or the signal for thymocyte selection induced through CD69L is triggered by molecules other than CD69 that are expressed by thymocytes.

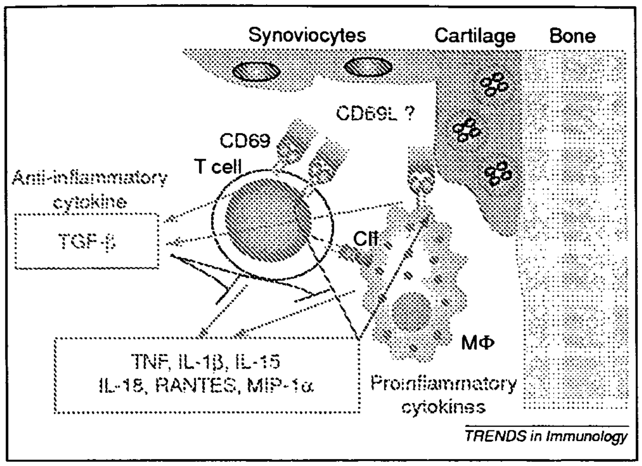

Figure 1. CD69 acts as an immunoregulatory molecule through the production of TGF-β. In collagen-induced arthritis, T cells are activated by collagen-derived peptides (CII) presented by macrophages (Mϕ), which induce CD69 expression and the release of proinflammatory cytokines. These cytokines and co-stimulatory molecules contribute to the persistent expression of CD69 in the inflammatory foci. Under such circumstances, the interaction of CD69 with its putative ligand(s) (CD69L) would induce the synthesis of the anti-inflammatory cytokine TGF-β, which reduces the secretion of proinflammatory cytokines and the activation of immune cells, thereby ameliorating tissue damage.

which might result in a pro- or anti-inflammatory state, conditioned by the particular environment.

Possible regulatory steps affected by CD69

Recent results indicate that CD69 modulates the synthesis of immunoregulatory molecules. Initial T-cell activation and antigen-driven T-cell proliferation are not affected by the absence of CD69 [21]. However, CD69 might affect the immune response during T-cell differentiation (Figure 2), involving immunoregulatory cytokines that include, but might not be limited to, TGF-β, which controls T-cell differentiation [31] and that, depending on the stimulation provided, could also regulate proinflammatory molecules.

CD69 is persistently expressed *in vivo* by T cells under certain conditions characterized by chronic inflammation [22], and *in vitro* on constant stimulation with proinflammatory cytokines or through certain adhesion receptors [15,34]. As stated earlier, the CIA model in CD69-deficient mice shows that local TGF-β levels in the joint are reduced [25], suggesting that in wildtype mice this receptor would interact with its putative ligand(s), inducing TGF-β, and thus dampen the local immune response (Figure 1). In this regard, TGF-β is found in the synovial fluid from rheumatoid arthritis (RA) patients [35], where it might counterbalance the activity of proinflammatory cytokines. In addition, the presence of IgG anti-CD69 autoantibodies, detected in the serum of a subset of RA patients, correlates with disease severity [36]. Therefore, we can hypothesize that these autoantibodies are able to block the interaction of CD69 with its putative ligands, decreasing TGF-β production and resulting in more severe disease. Alternatively, these autoantibodies could enhance signalling through CD69, generating proinflammatory mediators.

The induction of TGF-β synthesis through CD69 ligation might also take place in lymph nodes during the antigen-induced T-cell differentiation (Figure 2). In this regard, it is well known that CD69 is transiently expressed during T-cell activation and differentiation following antigen presentation by dendritic cells (DCs), and it is feasible that CD69 could exert a negative regulatory activity through TGF-β production. TGF-β downregulates antigen-presenting cell (APC) function [37,38] and limits activation and polarization of T cells to a Th1 or Th2 phenotype [31] (Figure 2). Therefore, enhanced T-cell differentiation in the absence of CD69 could explain the stronger effector response [25]. This negative regulatory role for every CD69-expressing T cell would normally lead to a non-specific limitation of the immune response. However, CD69 might mediate such role only in specific circumstances, for example, CD69 might be upregulated under tolerogenic conditions in the absence of other activating molecules that might counteract the negative effect of CD69. Alternatively, the expression of CD69 ligands under tolerogenic but not immunogenic conditions could limit the regulatory function exerted through CD69. Finally, other cell types with a variety of functional subsets, including monocytes and DCs, might express CD69, leading to distinct outcomes depending on the specific cellular and pathophysiological setting in which CD69 is expressed.

CD69 and regulatory T cells

T regulatory (Treg) cells have an impaired capacity to respond to proliferative signals and are able to inhibit other immune cell functions through cell–cell contact or through the production of anti-inflammatory cytokines, such as TGF-β, IL-10 or IL-4 [39,40]. Natural Treg cells are generated in the thymus and are characterized by their high expression of CD25, which suppresses effector responses through cell–cell contact in a cytokine-independent manner. However, adaptive Treg cells are generated from mature T lymphocytes after antigenic stimulation in the periphery, show a variable expression of CD25, and their mechanism of suppression of effector T-cell responses is cytokine-dependent [41]. It is feasible that CD69 could be persistently expressed by a subset of these cells (Figure 2).

In a murine lupus model, a subset of $CD4^+CD69^+$ cells has been detected in peripheral lymphoid tissues and inflammatory infiltrates. These cells are anergic and unable to synthesize proinflammatory cytokines [42]. Moreover, these $CD4^+CD69^+$ cells inhibit cytokine synthesis by $CD4^+CD69^-$ cells in a process that seems to be dependent on TGF-β because it is inhibited by anti-TGF-β antibodies [42]. Interestingly, peripheral blood mononuclear cells from lupus patients show an increased expression of CD69 [43] and the poor *in vitro* response of these cells to different stimuli is well known. Likewise, freshly isolated human synovial fluid T cells display a profound state of hypo-responsiveness that correlates with the expression of CD69 [44]. Therefore, some T cells bearing CD69 appear to possess the two main characteristics of Treg cells, namely their anergic behavior and their regulatory role. However, it is not currently known whether only a subset of the entire population of CD69-expressing cells is able to synthesize TGF-β *in vivo* and acts as a regulatory cell subset in different chronic

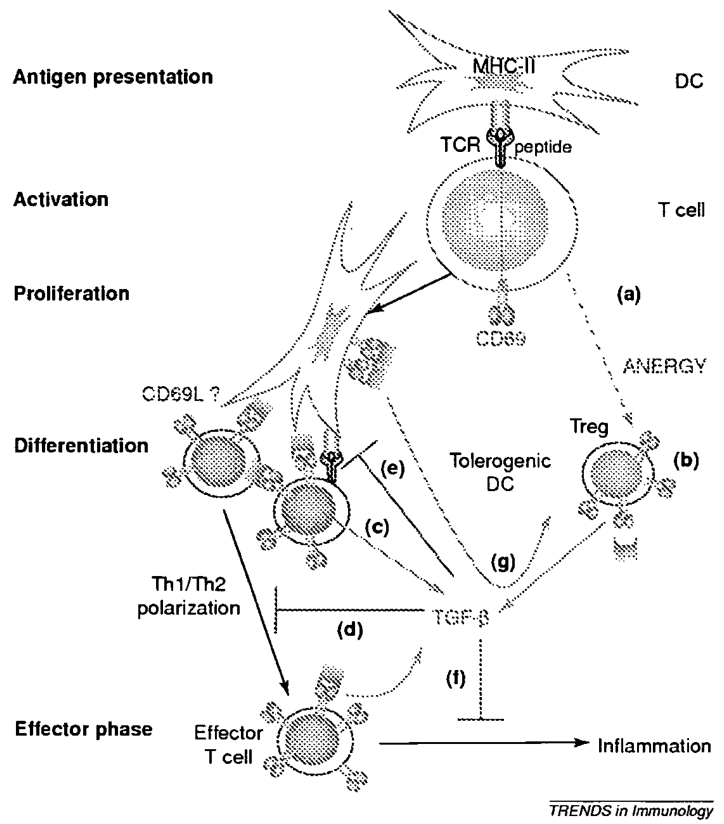

Figure 2. CD69 might be involved in multiple regulatory steps during the immune response. The antigen-specific signal activates T cells through the TCR and induces CD69. (a) However, incomplete activation might lead to anergy. (b) Some of these anergic lymphocytes might act as Treg cells and CD69 could act through TGF-β production as a survival factor for this cell subset. Alternatively, if the balance of the second signal is positive, T lymphocytes will proceed to cell proliferation. In this case, CD69 might exert its immunoregulatory effect at two different levels. First, if CD69L were expressed by DCs at lymph nodes, (c) $CD69^{+}$ T cells might produce TGF-β, a cytokine that inhibits both (d) T-cell differentiation (Th1 and Th2 polarization) and (e) APC function. (f) Second, if CD69L is expressed at inflammatory cell infiltrates, leukocytes persistently expressing high levels of CD69 would produce TGF-β, which would dampen inflammation. (g) Finally, CD69 expression might define a subset of tolerogenic DCs.

inflammatory conditions [42–44]. It is expected that CD69 should have an essential role in the proper function of these cells. TGF-β induces FoxP3 and a regulatory phenotype in TCR-challenged $CD4^{+}CD25^{-}$ naïve T cells [45] and this might also explain that, under certain circumstances, the CD69-dependent induction of TGF-β could influence the development of a subset of adaptive Treg cells.

Concluding remarks

Previous results *in vitro* pointed to CD69 as a stimulatory receptor, however, recent results *in vivo* have shown that the behaviour of CD69 is more complex. The absence of CD69 leads to an enhanced immune response in two independent models: increased severity of a T-cell driven animal arthritis model [25] and augmented rejection of NK-sensitive tumours [27]. CD69 mediates TGF-β production and the effect of this pleiotropic cytokine might account for the regulatory effect of CD69, although other mediators could be involved. CD69 could affect different steps in the distinct mechanisms responsible for the limitation of immune responses. First, CD69 might have a role in the deletion of lymphocytes by apoptosis following activation [27]. Second, during antigen presentation, partial activation signals might be able to simultaneously induce CD69 expression [15,34] and an anergic state, which is a characteristic of CD69-expressing lymphocytes in chronic inflammatory diseases [42,44]. Conceivably, these anergic lymphocytes could correspond to an as yet poorly defined adaptive Treg-cell subset that could act as a bystander suppressor lymphocyte population through the production of TGF-β or other immunoregulatory cytokines, mediating localized or systemic immune deviation [42,46]. Third, CD69 engagement might regulate the final balance of Th1/Th2 differentiation. The characterization of CD69 ligand(s), and the knowledge of their spatial and temporal expression, will shed further light on the precise immunoregulatory functions of CD69.

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